Vascular outcomes and developmental programming in a mouse model of sleep apnea

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Mohammad Badran

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the dissertation entitled:

**Vascular outcomes and developmental programming in a mouse model of sleep apnea**

Submitted by Mohammad Badran in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmacology.

**Examiing Committee:**

Dr. Ismail Laher  
Supervisor

Dr. Najib Ayas  
Co-supervisor

Dr. Vincent Duronio and Dr. Angela Devlin  
Supervisory Committee Member

Dr. Stephanus Van Eeden  
University Examiner

Dr. Brian Rodrigues  
University Examiner
Abstract

Obstructive sleep apnea (OSA) is a chronic condition characterized by recurring upper airway collapse during sleep, leading to chronic intermittent hypoxia (CIH) that can evoke oxidative stress and inflammation leading to cardiovascular disease (CVD). Current treatments for OSA are relatively ineffective in preventing CVD. Moreover, the effects of gestational OSA on the health of the offspring are unknown. We hypothesized that 1) antioxidant treatment can improve vascular outcomes in mice exposed to CIH and that 2) gestational intermittent hypoxia (GIH) can adversely impact fetoplacental outcomes and lead to cardiometabolic disease in the adult offspring.

The first chapter of this thesis examines the effects of CIH on vascular function, oxidative stress and inflammatory markers in CB57BL/6 male mice with or without treatment with the dietary antioxidant, alpha lipoic acid (ALA). CIH impaired aortic relaxation and basal nitric oxide (NO) production. Furthermore, CIH increased systemic oxidative stress, inflammation and proinflammatory gene expression in the aorta. Treatment with ALA improved endothelial function and reduced oxidative stress and inflammation.

In the second chapter, the impact of 14.5 days of GIH on vascular function of pregnant mice is reported. The following were evaluated: uterine artery function, plasma oxidative stress and inflammatory markers, spiral artery remodeling, placental morphology, hypoxia, oxidative stress, and fetal weights. GIH increased placental weights and decreased fetal weights, impaired uterine artery function, increased systemic oxidative stress and inflammation, increased placental hypoxia, and oxidative stress with no effect on spiral artery remodeling.
In the third chapter, aortic endothelial and perivascular adipose tissue (PVAT) function were evaluated in sixteen-week-old offspring of dams exposed to GIH only *in utero*. GIH male offspring had increased body weights and developed metabolic syndrome. Furthermore, aortic relaxation was impaired in offspring with a loss of PVAT anti-contractile effects, which was facilitated by adiponectin. Levels of adiponectin were lower in the PVAT and in plasma. Pyrosequencing of adiponectin promoter in PVAT indicated increased DNA methylation in male GIH offspring.

These data suggest that treatment of OSA patients with ALA could be a strategy to improve cardiovascular outcomes. Furthermore, maternal OSA may lead to adverse metabolic and vascular outcomes during adulthood.
Lay Summary

Obstructive sleep apnea (OSA) is a sleep disorder characterized by repetitive events of intermittent hypoxia (IH). This condition can trigger excessive production of oxidants and inflammatory substances that can be harmful to our body systems. OSA has been linked to the development of cardiovascular disease (CVD) and current treatments for OSA have been found to be both inconvenient and ineffective. The findings in this thesis can contribute significantly to the growing area of OSA research and the unknown cardiovascular effects of OSA in pregnant women and their offspring. This project investigated the use of an antioxidant alpha lipoic acid (ALA), and how it may protect against CVD in OSA patients. We determined that ALA can be used as a preventive measure and hence treatment for blood vessel dysfunction, a known consequence of IH. Our findings also inform the potential adverse effects of OSA in pregnant women and their offspring.
Preface

All of the work presented in this thesis was conducted in the Department of Anesthesiology, Pharmacology & Therapeutics at the University of British Columbia, Point Grey campus, and was approved by the University of British Columbia’s Animal Care Committee [certificate # A16-0291 and A17-0140].

In Chapter 1; Figures 1-2, 1-10, and Table 1-6 are used or modified with permission from applicable sources. Figure 1-1, 1-4, 1-5, and Table 1-1, 1-2, 1-4 and portions from the introductory text are used or modified with permission from Golbidi et al. (2011), Golbidi et al. (2012), Badran et al. (2014), Badran et al. (2015) of which I am the lead author or co-author.

A version of Chapter 2 is currently under review, as a manuscript titled “Alpha lipoic acid improves endothelial function and oxidative stress in a mouse model of sleep apnea”. I was the lead investigator of this chapter, responsible for all major areas of concept formation, data collection as well as manuscript writing. B. Abu Yassin and Dr. S. Golbidi provided technical support while Dr. I. Laher and Dr. N.T. Ayas aided in the concept of the study, revised and approved the manuscript for submission.

A version of Chapter 3 is currently under review, a manuscript titled “Gestational intermittent hypoxia impairs uterine artery function in pregnant mice”. I was the lead investigator of this chapter, responsible for experiment design, data collection and analysis, and manuscript writing. B. Abu Yassin and Dr. S. Golbidi provided technical support while Dr. I. Laher and Dr. N.T. Ayas aided in the concept of the study, revised and approved the manuscript for submission.
A version of Chapter 4 is currently under review, a manuscript titled “Gestational intermittent hypoxia induces endothelial dysfunction, reduces perivascular adiponectin and causes epigenetic changes in adult male offspring mice”. I was the lead investigator of this chapter, responsible for experiment design, data collection and analysis, and manuscript writing. Dr. D. Lin from Dr. Kobor lab at British Columbia Children’s Hospital Research Institute performed pyrosequencing for DNA methylation. B. Abu Yassin and Dr. S. Golbidi provided technical support while Dr. I. Laher and Dr. N.T. Ayas aided in the concept of the study, revised and approved the manuscript for submission.
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List of Abbreviations

4-HNE: 4-hydroxynonenal
8-OHdG: 8-hydroxy-2’-deoxyguanosine
ACh: acetylcholine
ADMA: asymmetric dimethylarginine
ADRF: adipocyte-derived relaxing factor
AdR1: adiponectin receptor-1
AHI: apnea-hypopnea index
ALA: alpha lipoic acid
ALDH2: aldehyde dehydrogenase 2
AUC: area under the curve
BAT: brown adipose tissue
BH4: tetrahydrobiopterin
CIH: chronic intermittent hypoxia
CK7: cytokeratin 7
CPAP: continuous positive airway pressure
CRP: C-reactive protein
CVD: cardiovascular disease
DDAH2: dimethylarginine dimethylaminohydrolase 2
DHE: dihydroethidium
DNMT: DNA methyltransferases
ELISA: enzyme-linked immunosorbent assay
\( \text{E}_{\text{max}} \): maximal response

eNOS: endothelial nitric oxide synthase

FBG: fasting blood glucose

FBI: fasting blood insulin

FFA: free fatty acids

\( \text{FiO}_2 \): fraction of oxygen inspired

FMD: flow-mediated dilation

gAd: globular adiponectin

Gpx3: glutathione peroxidase 3

GHT: gestational hypertension

GIH: gestational intermittent hypoxia

GSH: glutathione

\( \text{H}_2\text{O}_2 \): hydrogen peroxide

HIF-1\( \alpha \): hypoxia inducible factor-1\( \alpha \)

Hmox1: heme oxygenase 1

IA: intermittent air

IALA: intermittent air lipoic acid

IARD: intermittent hypoxia regular diet

ICAM-1: intercellular adhesion molecule-1

Ifng: interferon gamma

IH: intermittent hypoxia

IHLA: intermittent hypoxia lipoic acid
IHRD: intermittent hypoxia regular diet

IL: interleukin

ITT: insulin tolerance test

IUGR: intrauterine growth retardation

L-NAME: $N_\omega$-nitro-L-arginine methyl ester

Mcp1: monocyte chemoattractant protein 1

NADPH oxidase: nicotinamide adenine dinucleotide phosphate oxidase

NF-κB: nuclear factor kappa-B

NO: nitric oxide

NOX: nicotinamide adenine dinucleotide phosphate

Nqo1: NADPH dehydrogenase

Nrf2: nuclear factor-erythroid 2-related factor 2

$O_2^-$: superoxide anion

ONOO$: peroxynitrite

OSA: obstructive sleep apnea

PKC-ε: -protein kinase C-epsilon

PAS: periodic acid–Schiff

PE: preeclampsia

Ph: phenylephrine

PIGF-2: placental growth factor

PVAT: perivascular adipose tissue

PVRF: perivascular-derived relaxing factor
RAAS: renin-angiotensin-aldosterone system

ROS: reactive oxygen species

RNS: reactive nitrogen species

SDB: sleep disordered breathing

sEng: soluble endoglin

sFlt1: soluble vascular endothelial growth factor 1

SMA: smooth muscle actin

SNP: sodium nitroprusside

TGF-β: transforming growth factor-β

TNF-α: tumor necrosis factor-α

TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling

VCAM-1: vascular cell adhesion molecule-1

VEGF: vascular endothelial growth factor

VSMC: vascular smooth muscle cell

VWAT: visceral white adipose tissue

U-46619: 9,11-dideoxy-9a,11a-methanoepoxy prostaglandin F₂α
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I dedicate this work to my father, Salah Al Din Badran, who passed away when I was young. He loved education and did the impossible to make sure his children attend the best schools despite his tough financial situation. I am certain he is looking down at me from the Heavens feeling proud that one of his children got this far.
Chapter 1: Introduction

1.1 Obstructive Sleep Apnea

Sleep is an integral part of life, with humans spending approximately 30% of their lives asleep. Poor sleep quality and reduced amounts of sleep can result in daytime sleepiness, decreased alertness, and reduced mental functioning. However, it is increasingly recognized that pathologic disruption of sleep and reduced amounts of sleep could also have substantial adverse cardiovascular outcomes (1). The most common respiratory sleep disorder by far is OSA; in subjects with OSA, breathing is interrupted because of recurrent collapse of the upper airway during sleep caused by sleep-induced loss in upper airway tone superimposed on an anatomically susceptible upper airway (2). The severity of OSA can be defined according to the apnea hypopnea index (AHI), which is the number of times the airway narrows (hypopnea) or collapses (apnea) per hour of sleep; by consensus, the AHI categorizes patients with sleep apnea into 3 groups: mild (>=5 and <15 events/h), moderate (>=15 and <30 events/h), and severe (>= 30 events/h) (3). Characteristic symptoms of OSA include loud snoring and witnessed apneic episodes. The recurrent episodes of apnea and hypopnea lead to arousal, sleep fragmentation, hypoxemia, and hypercapnia. Poor sleep quality leads to reduced vigilance, daytime sleepiness, and an increased risk of motor vehicle accidents (4). Furthermore, the recurrent arousals and hypoxemia/reoxygenation stimulates the sympathetic nervous system, oxidative stress, acute increases in blood pressure, and activation of systemic inflammation (5). These are some of the potential pathogenic mechanisms whereby OSA can lead to an increased risk of CVD (Figure 1-1).
The gold standard for the diagnosis of OSA is overnight polysomnography (PSG). This entails an overnight stay during which the patient is continuously monitored in an attended setting, with collection of a variety of physiological signals, including electrical activity using electroencephalography, eye movements, oxygen saturation, heart rate, respiratory excursion, and airflow (3). Portable unattended monitoring is being used increasingly, especially in the context of subjects with a high pretest probability of disease. These studies often have more limited channels (eg, no electroencephalographic signals) and lack the sensitivity and specificity of full PSG; however, they are less costly and may be more convenient for patients (6).

Figure 1-1: Mechanistic links between OSA and cardiovascular disease.
(Used with permission ©Badran et al. Epidemiology of Sleep Disturbances and Cardiovascular Consequences. Canadian Journal of Cardiology. 2015 Jul 1;31(7):873-9)
1.1.1 Prevalence of OSA

Although estimates of the prevalence of OSA vary (largely because of differences in the methods and definitions used), it is clear that OSA is common and underdiagnosed. From a community-based study of middle-aged (30-60 years) men and women performed in 1988 in Wisconsin, it is estimated that approximately 24% of men and 9% of women have OSA (defined as an AHI > 5 events/h) and that 9% of men and 4% of women have moderate to severe OSA (i.e., AHI > 15 events/h) (7). The increased prevalence of obesity, defined as a body mass index (BMI) ≥ 30, over the past 2 decades have set the current estimated OSA prevalence as being 14%-55% higher (8). Similar prevalence estimates occur in other geographic regions, including Europe and Asia (9, 10). The prevalence increases with age; Ancoli-Israel et al. studied elderly individuals aged 65 years using portable sleep studies, and report that 62% of participants had 10 or more episodes of apnea and hypopnea per hour of sleep (11).

In 1997, it was estimated that nearly 82% of men and 92% of women with moderate or severe OSA were not clinically diagnosed (12). Although the percentage of undiagnosed subjects may now be less, the undiagnosed population of OSA remains very high in both North America and other countries (13). Given the high prevalence of disease and associated adverse health and safety outcomes, the societal economic costs of OSA in Canada are likely in the billions of dollars per year (14).
1.1.2 Risk Factors for OSA

There are many risk factors for OSA, with the strongest modifiable risk factor being obesity, especially central obesity (e.g., neck circumference); nearly 60%-90% of patients with OSA are obese (4). There are multiple potential mechanisms whereby obesity could lead to OSA, including narrowing of the upper airway due to fat deposition, reduced upper airway function, and reduction in lung volumes leading to a less stable upper airway (15). Longitudinal studies have shown that a 10% increase in weight was associated with a 6-fold increased risk of development of OSA during a 4-year follow-up period, whereas a 10% weight loss was associated with a 26% decrease in AHI (95% confidence interval [CI], 18%-34%) (16). Interventions to promote weight loss improve OSA and should be recommended in obese subjects (17). Although obesity is likely a cause of OSA rather than vice versa, some have argued a potential bidirectional association with OSA, perhaps resulting from metabolic dysregulation or fatigue contributing to a lack of activity with OSA and potentially contributing to weight gain (17).

Men have approximately twice the prevalence rate of OSA as women. There are a number of factors that could account for the difference in prevalence between the sexes. Some of these may be structural in nature, e.g., upper airway fat deposition might be greater in men than in women because men tend to have predominantly upper body fat, whereas women tend to have lower body fat distribution. Female and male hormones may also play roles. Postmenopausal women have a 2- to 3-fold increased risk of OSA compared with premenopausal women, which is not accounted for by body mass index, age, or other risk
factors (18), and women with polycystic ovary disease also have an increased rate of OSA (19). Male hormones also have an impact on sleep-disordered breathing. In a randomized clinical trial of 67 obese men with OSA, 1000 mg of testosterone undecanoate administered at 0, 6, and 12 weeks worsened OSA severity after 7 weeks in hypogonadal men when compared with placebo. After 18 weeks, AHI was essentially the same in both groups, and although the oxygen desaturation index was slightly greater (by 4.5 events/h), this was no longer statistically significant. This suggests that testosterone at these doses has only a temporary effect on OSA severity (20, 21). Certain ethnic groups may also have increased rates of OSA, with African Americans tending to be at increased risk (22). Asians have a prevalence rate of OSA similar to that of whites despite being less obese, which may be explained by differences in craniofacial structure (23). One potential risk factor that has been recently appreciated and may be of particular interest to cardiologists is upper airway oedema caused by fluid redistribution at night (24). Patients, particularly those in a fluid-retaining state (such as heart failure), may experience fluid retention during the daytime, with rostral redistribution when lying down during sleep. The consequent increased upper airway edema may then narrow the airway and predispose it to collapse, potentially worsening OSA severity. Other risk factors for OSA include family history/genetics (25, 26), smoking (27), alcohol (28), increased age, use of sedatives and muscle relaxants (29), upper airway structural abnormalities (eg, enlarged tonsils), hypothyroidism (30), and acromegaly (31); the latter 2 of which can affect both upper airway soft tissue and respiratory control.
Patients with OSA have a high prevalence of cardiovascular risk factors. As already mentioned, male sex, obesity, and increased age are risk factors for OSA. About 50% of patients with OSA have hypertension (32). Even though hypertension is prevalent in patients with OSA, whether OSA is an independent risk factor for hypertension is still open to debate. In the Wisconsin sleep cohort, subjects with an AHI of 15 events/h or more had a 3-fold increased risk of incident hypertension developing during 4 years study (33). In contrast, in the Sleep Heart Health Study, the independent association of OSA with hypertension was small and not statistically significant (33, 34). Continuous positive airway pressure (CPAP) therapy reduces blood pressure, although the effects are relatively modest (about 2-3 mm Hg overall). However, the impact is greater in subjects with more severe OSA and greater CPAP compliance (35–37).

The prevalence of type II diabetes in patients with OSA is high, ranging between 15% and 30%, depending on the methods used to diagnose both diseases (38). Several studies have also reported a significant association between increasing severity of OSA and the prevalence of diabetes. Whether OSA per se worsens glycemic control is unclear because obesity is common in patients with OSA. However, animal and human studies have shown negative impacts of intermittent hypoxia on glucose tolerance (39, 40). In some studies, treatment of OSA with CPAP improved glycemic control, but other studies have not reproduced these findings (41).
Similarly, individuals with OSA have a high rate of hyperlipidemia, but it is again unclear if this is from OSA per se or is attributable to the high rate of obesity (42, 43). Animal studies have demonstrated adverse effects of intermittent hypoxia on lipid metabolism (44), and human intervention studies using CPAP therapy have shown a potential improvement in hyperlipidemia (45). For example, Phillips et al. studied 29 subjects in a randomized crossover design (2 months of CPAP and 2 months of sham CPAP); use of CPAP resulted in a significant improvement in postprandial triglyceride and total cholesterol levels (46).

1.1.4 CVD in OSA

The prevalence of OSA among hospitalized men with acute myocardial infarction is nearly 70\% (47). In addition, multiple studies have demonstrated an independent relationship between incident CVD and OSA. For example, Marin et al. studied 1300 men who underwent PSG in the sleep laboratory and were followed over a mean of 10 years for fatal cardiovascular events (death from myocardial infarction or stroke) and nonfatal cardiovascular events (nonfatal myocardial infarction, nonfatal stroke, coronary artery bypass surgery, and percutaneous transluminal coronary angiography) (48). Men with untreated severe OSA had a significantly increased risk of incident fatal (odds ratio [OR], 2.87; 95\% CI, 1.17-7.51) and nonfatal (OR, 3.17; 95\% CI, 1.12-7.51) events, even after controlling for a variety of potential confounders (48). The rate of events was not significantly greater in subjects with untreated mild to moderate OSA after adjustment for confounders. Interestingly, subjects treated with CPAP had a markedly reduced risk of events that was similar to subjects without OSA, suggesting a potential therapeutic benefit.
of CPAP. In a subsequent study, similar associations of OSA, CPAP use, and cardiovascular events were found in women (49). One major limitation of these 2 studies was their observational nature, making it difficult to exclude residual confounding. This may especially be a limitation with respect to the CPAP data, because subjects who are adherent with CPAP may be different in many other ways than subjects who are not compliant or who are not offered therapy (e.g., may be more adherent with other medications, diet, and exercise).

Recently, a decade-long historical cohort study was published using clinical and health administrative data (50). Adults suspected of having OSA who were referred for PSG between 1994 and 2010 were followed until 2011 using health administrative data to assess the occurrence of a composite outcome (myocardial infarction, stroke, heart failure, revascularization procedures, and death). Over a median of 68 months of follow up, 1172 (11.5%) of 10,149 subjects had the composite outcome. OSA-related variables that were significant independent predictors of events included sleep time (4.9 vs 6.4 hours; hazard ratio (HR), 1.20; 95% CI, 1.12-1.27), time spent with oxygen saturation < 90% (9 vs 0 minutes; HR, 1.50) and daytime sleepiness (HR, 1.13; 95% CI, 1.01-1.28). AHI was significantly associated with a composite cardiovascular outcome, but after controlling for potential confounding factors, the association became nonsignificant. These data suggest that sleep breathing parameters other than AHI (e.g., nocturnal hypoxemia) might be more robust predictors of CVD. The major limitation of this study was the lack of information about treatment. However, some studies have not been able to demonstrate an independent association of OSA with CVD. In the Sleep Heart Health Study, which is a community-based cohort of participants enrolled in a variety of cardiovascular epidemiologic studies, > 4000
individuals participated in home sleep studies and were followed over time. After a median of 8.7 years of follow-up, the association between OSA and incident CVD was inconsistent and was only significantly associated with CVD in men younger than 70 years of age. The reason for the difference in results in this study as opposed to others is open to speculation. Perhaps the source of the subjects (clinic vs. community-based cohort) might have accounted for the differences in results (51). Subjects with atrial fibrillation have an increased prevalence of OSA (52). The presence of OSA in with atrial fibrillation may also be a risk factor for stroke. In a recent study, investigators studied > 300 subjects with atrial fibrillation; stroke risk was significantly greater in subjects with OSA (25% vs 8%). The increased risk persisted after controlling for a variety of potential confounders (OR, 3.65; 95% CI, 1.25-10.62) (53).

1.1.5 CPAP treatment in OSA

The mainstay treatment for OSA is CPAP and involves the use of a pump to deliver air into the mouth or nose via a mask during sleep. The airflow generates positive pressure that maintains the airway in an open state by preventing the soft tissue from collapsing. It has been proven that CPAP treatment can reverse daytime sleepiness and nocturnal symptoms, improve cognitive impairment, and prevent motor vehicle crashes in OSA patients (54–57). Studies have also shown that CPAP can have positive impact on cardiometabolic outcomes such as decreased diurnal blood pressure (58, 59), reduce risk of fatal and non-fatal cardiovascular events (48), improves insulin sensitivity (60) and dyslipidemia (61), although the long term effects of CPAP on those parameters is still inconclusive. On the other
hand, The SAVE study assigned 2717 eligible adults between 45 and 75 years of age who had moderate to severe OSA and coronary or cerebrovascular disease to receive CPAP treatment plus usual care or usual care alone. The mean duration of CPAP adherence was 3.3 hours per night and the AHI index decreased from 29 events/hour to 3.7 events/hour during follow up. After a mean of 3.7 years follow-up, the primary end point (death from CVD, myocardial infarction, stroke, heart failure, transient ischemic attack, or hospitalization for unstable angina) occurred in 229 participants (17%) treated with CPAP and in 207 participants (15.4%) who received usual care alone (HR with CPAP 1.10; CI 95%, 0.91-1.32, p = 0.34) (Figure 1-2) (62). This data shows that prescription of CPAP plus usual care, compared to usual care alone, did not prevent cardiovascular adverse outcomes in subjects with moderate to severe OSA. Moreover, in a recent meta-analysis, CPAP use was not associated with reduced risk of major adverse cardiac events (relative risk (RR) 0.94; CI 95%, 0.78-1.15) except in the subgroup that used CPAP >4 hours (RR 0.7; CI 95%, 0.52-0.94, p = 0.02). This data indicates that utilization of CPAP in subjects with OSA is not associated with improved cardiac outcomes unless patient uses CPAP for 4 hours duration or more (63). Indeed, OSA patient’s compliance to CPAP therapy is very low. Rotenberg et al. performed a systematic review on 82 papers and found that overall CPAP non-adherence based on 7-hours/night-sleep time over twenty years was 34.1% with no significant improvement over time (64). This data combined provides evidence that CPAP treatment is not only ineffective, but also undesirable, requiring immediate research into other interventions.
Figure 1-2: Cumulative event of the primary end point.

Shown is the cumulative incidence of a first primary end point (a composite of death from cardiovascular causes, myocardial infarction, stroke, or hospitalization for heart failure, unstable angina, or transient ischemic attack) in the group that received CPAP plus usual care (CPAP group) and in the group that received usual care alone (usual-care group). The inset shows the same data on an enlarged y axis. (Reproduced with permission from McEvoy et al. CPAP for prevention of cardiovascular events in obstructive sleep apnea. New England Journal of Medicine. 2016 Sep 8;375(10):919-31. Copyright Massachusetts Medical Society ©)
1.1.6 Animal models of OSA

OSA patients usually have comorbidities such as obesity, diabetes, or hypertension that likely will affect cause-effect relationships (2). Creating animal models of OSA would minimize the influence of comorbidities and behavioural variables common in humans. Using animal models also permits the use of pharmacological agents to study the pathological mechanisms under a well-controlled environment. Ideally, animal models should mimic OSA in humans in at least three ways: (i) they share aspects of the underlying pathophysiology, (ii) have similar symptoms and the spectrum of disease severity that occur in humans, and (iii) respond to treatment modalities that are useful in humans. Furthermore, a short life span (to allow for the unveiling of a wide range of disease-related complications within a reasonable time period), routine availability, cost effectiveness, and availability of disease-free littermates add to the usefulness of animal models. There are additional considerations when using animals that need to be considered for sleep-related research. Animal models for studying sleep-disordered breathing should address at least one (or a combination) of the three main injurious consequences of sleep apnea: intermittent hypoxia/hypercapnia, strained breathing due to mechanical obstruction, and sleep fragmentation (5). In this regard, rodents are amenable to genetic manipulation suitable for the production of phenotypes that may characterize OSA in humans. One advantage of using rodent models to examine neurophysiological aspects of sleep apnea in humans is the high degree of similarity between the structures of the nervous systems of rodents, such as rats and mice and humans.
A useful animal model of OSA is the English bulldog, since no surgical interventions or genetic manipulations are required. There is a strong resemblance in sleep apnea between humans and English bulldogs, making this animal model a suitable candidate for experimental use. It was noticed that these dogs snore and have hypopneas and frequent arousals from sleep, mainly due to an abnormal upper airway anatomy characterized by an enlarged soft palate and a narrowing of the oropharynx. These animals have episodes of both central and obstructive apnea with haemoglobin desaturation (<90%) that worsens during rapid eye movement (REM) sleep and is accompanied by daytime hypersomnolence as evidenced by shortened sleep latency (65). Alterations in the contractility of respiratory muscles were first reported in genetically obese Zucker fat rats (ZFR) in 1996 (66). These animals show many of the cardiopulmonary deficits described in obese humans, such as respiratory control dysfunction, chest wall limitation, upper airway narrowing, hypertension, myocardial hypertrophy, and poor exercise capacity (67–69). Later studies suggested that these rats also exhibit signs of sleep apnea (70). OSA can also be stimulated through surgical procedures that induce airway obstruction (71). This procedure has mainly been used in larger animals such as dogs (71, 72), piglets (73), baboons (74), and small rodents (75, 76). Studies have incorporated sophisticated apparatus to detect sleep-wake states so that initiation of airway obstruction could be coordinated with sleep onset (77). The most commonly used animal model in the area of OSA is the intermittent hypoxia (IH) model. This murine model represents extreme physiological changes occurring during sleep-related IH and was first described in 2001 by Tagaito et al. (78). Mice are housed in customized cages to deliver either an intermittent hypoxic stimulus or an intermittent room
air control. Ports evenly spaced near the bottom of the cages allow gases to enter from four sides at the level of the bedding material. A gas control delivery system regulates the flow of room air, N₂, and O₂ into the customized cages housing the mice. Programmable solenoids and flow regulators control the manipulation of inspired O₂ fraction (FiO₂) levels in each cage over a wide range of IH profiles. During the 12-h light cycle, FiO₂ is reduced from 20.9 to 5.0% over a 30-s period and rapidly reoxygenated to room air levels using a burst of 100% O₂ during the following 30-s period. During the 12-h dark cycle, a constant flow of room air is delivered to the cages. The use of multiple inputs into the cage produces a uniform nadir FiO₂ level throughout the cage (Figure 1-3).

Figure 1-3: Illustration of intermittent hypoxia model in rodents.
(Original figure)
Table 1-1: Selected animal models of sleep apnea with some of the related benefits and disadvantages

<table>
<thead>
<tr>
<th>Model</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
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<tbody>
<tr>
<td>Natural (spontaneous) models of SDB</td>
<td></td>
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</tr>
<tr>
<td>English bulldog</td>
<td>- No intervention needed for inducing sleep apnea</td>
<td>- Significant hypoxia occurs only during REM sleep</td>
</tr>
<tr>
<td></td>
<td>- Shows both central and peripheral aspects of sleep apnea</td>
<td>- Milder NREM disease (apnea) in bulldogs (AHI &lt; 15) compared to humans</td>
</tr>
<tr>
<td></td>
<td>- Similar pathogenesis with human disease in terms of chronicity and progression</td>
<td>- Apnea is not related to obesity</td>
</tr>
<tr>
<td></td>
<td>- Similar polysomnographic structure during NREM sleep in bulldogs and patients with upper airway resistance syndrome</td>
<td>- Large animals are expensive to use in research</td>
</tr>
<tr>
<td>Obese Yucatan miniature pigs</td>
<td>- Apnea is obesity-related</td>
<td>- Majority of apnea/hypopnea events occur during REM sleep</td>
</tr>
<tr>
<td></td>
<td>- Shows both central and peripheral aspects of sleep apnea</td>
<td></td>
</tr>
<tr>
<td>Obese Vietnamese pot-bellied pig</td>
<td>- Apnea is obesity-related</td>
<td>- Not suitable models for genetic studies</td>
</tr>
<tr>
<td></td>
<td>- Impaired baroreflexes make them suitable for studying the relationship between sleep apnea and blood pressure</td>
<td></td>
</tr>
</tbody>
</table>
### C57BL/6J mice
- They show two types of central apnea, post-sigh and spontaneous apnea
- Post-sigh apnea is sleep stage-dependent, similar to human children and adolescents, which makes it suitable for studying SIDS
- Genetic engineering easily applied to mice
- Transgenic mice can provide valuable information about molecular targets of sleep apnea

### Induced airway obstruction

| Induction of obstruction through endotracheal tube | Computerized machine can detect sleep/wake pattern and conduct obstruction during sleep hours | Some technical issues related to use in small animals
Need for surgical intervention |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Applying a latex collar around the neck</td>
<td>No need for surgical intervention</td>
<td>Presence of obstruction during waking hours</td>
</tr>
<tr>
<td></td>
<td>The degree of obstruction is adjustable</td>
<td>Problems of using large animals for research</td>
</tr>
<tr>
<td></td>
<td>Easy to use in rodents</td>
<td></td>
</tr>
</tbody>
</table>
| Induction of hypoxia by endotracheal tube or mask | - Allows evaluation of the effects of varying degrees of oxygen desaturation  
- Can compare the contribution role of hypoxia distinct from CO₂  
- Evaluation of different patterns of hypoxic cycle (intermittent vs. sustained) and exposure duration (acute vs. chronic) | - Needs surgical intervention or animal restraint  
- No airway obstruction and strenuous breathing |
| Induction of hypoxia by hypoxic tent or cage | - Same benefits as above model with added feature of being free of surgical intervention | - Does not allow for the study of potential strenuous breathing, making it unsuitable for studying the pathogenesis of OSA |

1.1.7 Mechanisms underlying CVD in OSA

1.1.7.1 Oxidative stress

Oxidative stress results from an imbalance between antioxidant defense mechanisms and the production of oxidants, meaning that either a decreased antioxidant capacity and/or overproduction of reactive oxygen and nitrogen species (ROS/RNS) causes oxidative stress (79). Although free radicals have important roles in regulating signal transduction and cellular function, their overproduction can damage lipids, proteins, and DNA, thus affecting many cellular and physiological mechanisms (80). Recent studies show important links between the hypoxia-related free radicals related oxidative stress and cardiovascular disease in OSA subjects (81).

1.1.7.1.1 Sources of Reactive Oxygen Species in OSA

Free radicals are atoms or molecules containing one or more unpaired electrons in their atomic or molecular orbitals and thus are chemically unstable and highly reactive. Usually when two radicals react, the product is a non-radical, but when radicals react with non-radicals the product is a new radical and, therefore, the radical chain reaction propagates (82). Oxygen metabolism during normal cellular respiration generates ROS as by-products, and their elimination occurs through enzymatic and non-enzymatic antioxidant systems. When ROS generation exceeds the capacity of antioxidants, oxidative stress and damage to cells and tissues ensue. This can contribute to pathological conditions of cardiovascular disease.

Oxygen has a unique electronic configuration; the addition of one electron to molecular oxygen can result in the production of the superoxide anion. Superoxide is considered the
primary ROS and can interact with other molecules to generate secondary ROS/RNS, either directly or through enzyme- or metal-catalyzed processes. Superoxide anions can give rise to the production of many toxic molecules such as hydrogen peroxide, hydroxyl radical and peroxynitrite (83). The latter is a RNS and results from a reaction between superoxide anion and nitric oxide (NO), an important endothelium-derived vasodilator. As a result, nitric oxide bioavailability decreases and the vasodilator ability of blood vessels is compromised (84).

Mitochondria are major sources of superoxide anion due to reactions occurring during oxidative phosphorylation. It is estimated that 3–5% of the oxygen consumed by mitochondria is converted to superoxide anion during aerobic respiration. During hypoxia, ROS production is elevated due to excessive mitochondrial reduction (82). NADPH oxidase is also a very important source of superoxide anion. Phagocytic cells contain this enzyme and other enzymes to produce ROS as a defense mechanism against pathogens. Although this mechanism can protect against invading microbes, it can also cause damage to surrounding tissue (85). NADPH oxidase is also expressed in non-phagocytic cells where it usually generates lower amounts of superoxide anion for purposes such as signaling (86). For example, NADPH oxidase is expressed in vascular cells where generation of superoxides plays an important role in vascular cell growth (87). Figure (1-4) shows the production of different ROS, their physiologic function and role in disease state.
**Figure 1-4: Reactive oxygen/nitrogen species produced during OSA/IH**


**1.1.7.1.2 Evidence of Oxidative Stress in OSA**

Many studies confirm the association of OSA with oxidative stress through measurements of oxidative stress markers. For example, Schulz et al. report increased production of superoxide anion in stimulated neutrophils and monocytes from OSA subjects.
(88), while others report that superoxide anion production was significantly higher in non-stimulated monocytes of OSA subjects (89, 90). Oxidative stress markers of lipid peroxidation, protein carbonylation and DNA oxidation are increased in OSA subjects. Lipid peroxidation is an important marker of oxidative stress since lipids are easily oxidized. In an overnight study of OSA subjects with and without cardiovascular disease, levels of thiobarbituric acid (TBARS), a marker of lipid peroxidation, were significantly increased (91). In another study, fourteen males with severe OSA fasted all night and TBARS levels were measured in the next morning. TBARS levels in those subjects were significantly higher compared to thirteen healthy age matched controls (92). Oxidized LDL is also increased in OSA, where plasma levels of oxidized LDL were higher in OSA subjects (43.6 U/L) compared to control (32.3 U/L) (92). Protein carbonylation (oxidation of protein side chain) is increased as well in subjects with moderate to severe OSA where protein carbonyl levels were significantly higher when compared to matched controls. On the other hand, the increase was not significant in mild OSA subjects (93). 8-hydroxyl-2′deoxyguanosine (8-OHdG), a marker of DNA oxidation, is also elevated in OSA subjects. Urinary excretion of 8-OHdG significantly correlates with the severity of OSA (81).

Evaluating oxidative damage in OSA subjects is essential since oxidative stress is one of the main causes of endothelial dysfunction. Yamauchi et al. studied 32 OSA and 15 control subjects, in which they quantified endothelial nitric oxide synthase (eNOS), phosphorylated eNOS (the active form of the enzyme responsible for producing NO in the vasculature), inflammation (cyclooxygenase-2 and inducible NOS), and oxidative stress (nitrotyrosine). They also evaluated vascular reactivity in these subjects by flow-mediated dilation (81).
Endothelial expression of eNOS and phosphorylated eNOS decreased by 59% and 94%, respectively, in untreated OSA subjects (n = 14, endothelial cells harvested by scraping the intima of a superficial forearm vein with a J-shaped endovascular wire). Nitrotyrosine and cyclooxygenase-2 expression was 5-fold greater in OSA subjects. In subjects who adhered to CPAP ≥ 4 hours a day (n = 14), the expression of nitrotyrosine, cyclooxygenase-2, and inducible NOS was decreased significantly, while CPAP treatment restored eNOS and phosphorylated eNOS expression levels with concomitant reduction in oxidative stress. Of interest is that the effect of CPAP may be restricted to limiting free radical production, as antioxidant defense mechanisms were unaffected. Flow-mediated dilation in OSA subjects was significantly decreased, but adhering to CPAP ≥ 4 hours a day significantly improved endothelial dependent vasodilation (94).

Antioxidant capacity is impaired in OSA subjects. Although the antioxidant capacity in OSA subjects and controls did not differ in their study, Christou et al. showed a linear negative relationship between antioxidant capacity and apnea/hypopnea index ($R = -0.551, p = 0.041$) (95). Total antioxidant status in OSA subjects is significantly decreased when compared to healthy subjects, with lower levels of vitamin A and E when compared to control (96). On the other hand, Katsoulis et al. reported some unexpected results where they found that total antioxidant status before and after sleep was significantly lower in OSA subjects with AHI < 30 but not in severe OSA subjects with AHI > 30. A possible explanation could be due to differences between the acute effects of hypoxia resulting from apneic sleep and chronic oxidative stress that may be sustained in severe OSA subjects even during the daytime (97).
### 1.1.7.2 Inflammation

Elevated levels of plasma C-reactive protein (98), leukocyte superoxide (89), and soluble adhesion molecules (99) are indicators of an underlying systemic chronic inflammatory response in OSA subjects. Many studies associate ROS molecules with the induction of a cascade of inflammatory pathways that cause an overexpression of adhesion molecules and proinflammatory cytokines (100). Adhesion molecules facilitate the recruitment and accumulation of leukocytes, platelets, and possibly red blood cells on the endothelial lining of the vasculature to further promote endothelial cell injury (100). There is an increased expression of adhesion molecules (such as CD15 and CD11c) and enhanced adherence of monocytes obtained from subjects with OSA in cultured human endothelial cells (89). Several years later the same group suggested a role for CD4 and CD8 T cells in atherogenesis and plaque formation in OSA subjects. By using flow cytometry and chromium release assays, they demonstrated that CD4 and CD8 T cells undergo phenotypic and functional changes and acquire more cytotoxic capabilities. Thus, there was a shift in CD4 and CD8 T cells toward type 2 cytokine dominance, with increased IL-4 (a proinflammatory cytokine) and decreased IL-10 (an anti-inflammatory cytokine) expression and an increased production of TNF-α (101). Increased levels of IL-6 and IL-8 in OSA subjects strongly correlate with the duration of hypoxic events (102). TNF-α is a proinflammatory cytokine that induces oxidative stress in endothelial cells, increases the expression of endothelial cell adhesion molecules, and induces cytokine production via activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB) resulting in endothelial dysfunction. TNF-α also induces the expression of NADPH oxidase, which potentially is a
source of ROS production, and in this way completes the vicious cycle of oxidative stress-inflammation and vice versa (103). TNF-α antagonists (etanercept, infliximab) have a favourable impact on daytime sleepiness in OSA subjects, an effect that is reportedly more effective than the benefit conferred by CPAP treatment (104).

1.1.7.3 Oxidative stress and inflammation in animals exposed to intermittent hypoxia

There is much support in the literature for the idea that oxidative stress is a consequence of intermittent hypoxia. Rats subjected to intermittent hypoxia for two weeks have increased vascular production of ROS (105). IH-induced pulmonary hypertension in mice leads to increased lung levels of the NADPH oxidase subunits NOX4 and p22phox, indicating that NADPH oxidase-derived ROS contributes to the development of pulmonary hypertension caused by chronic intermittent hypoxia (106). NADPH oxidase is activated in tissues such as the myocardium, brain, carotid body, and liver in various animal models of IH (107–109). As for oxidative stress markers, one month of IH significantly increased MDA levels in mice (110). This agrees with a study by Savransky et al. who reported that serum MDA levels increased 4-fold in mice subjected to chronic IH for 6 months when compared to control (111). Oxidative stress markers are also elevated in tissues such as the liver and brain (112, 113). Inflammatory markers were also increased in the plasma and other tissue in animals exposed to chronic intermittent hypoxia. Table 2 describes the intermittent hypoxia profile used in rodents and their influence on oxidative stress and inflammatory markers measured.
Table 1-2: Different biomarkers of oxidative stress and inflammation in rodent models of intermittent hypoxia

<table>
<thead>
<tr>
<th>Reference</th>
<th>Species</th>
<th>Hypoxia regimen</th>
<th>Measured marker</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>(110)</td>
<td>CD-1 mice (male)</td>
<td>8 min cycles of $FiO_2$ 8.5% and 21% for 30 days</td>
<td>MDA</td>
<td>↑</td>
</tr>
<tr>
<td>(114)</td>
<td>ApoE−/− mice (male)</td>
<td>30-s cycles of $FiO_2$ 6.5%–21% 8 h/day for 4 and 12 weeks</td>
<td>OxLDL</td>
<td>↑</td>
</tr>
<tr>
<td>(115)</td>
<td>C57BL/6J mice (male)</td>
<td>2 min 6% $O_2$ and 2 min 21% $O_2$ for 8 h/day for 1, 2, and 4 weeks</td>
<td>TBARS</td>
<td>↑</td>
</tr>
<tr>
<td>(112)</td>
<td>CF-1 mice (male)</td>
<td>30-s cycles of $FiO_2$ 8% 8 h/day for 21 and 35 days</td>
<td>DNA damage</td>
<td>↑</td>
</tr>
<tr>
<td>(115)</td>
<td>C57BL/6J mice (male)</td>
<td>2 min 6% $O_2$ and 2 min 21% $O_2$ for 8 h/day for 1, 2, and 4 weeks</td>
<td>Protein carbonyls</td>
<td>↑</td>
</tr>
<tr>
<td>(116)</td>
<td>C57BL/6J mice (male)</td>
<td>30-s cycles of $FiO_2$ 4.5%–21% 8 h/day for 10 days</td>
<td>NADPH-dependent</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>superoxide production</td>
<td></td>
</tr>
<tr>
<td>(105)</td>
<td>Sprague-Dawley rats (male)</td>
<td>90-s cycles of $FiO_2$ of 5%, 20 cycle/h, 7 h/day for 14 days</td>
<td>Qualitative measurement of superoxide anion</td>
<td>↑</td>
</tr>
<tr>
<td>(117)</td>
<td>Sprague-Dawley rats (male)</td>
<td>5-s cycles of $FiO_2$ 21% to 10% every 90 s for 4 weeks</td>
<td>SOD levels</td>
<td>↓</td>
</tr>
<tr>
<td>(118)</td>
<td>Wistar rats (male)</td>
<td>30-s cycles of $FiO_2$ 21% to 5% for 6 weeks</td>
<td>TNF-α, ICAM-1, IL-10</td>
<td>↑</td>
</tr>
<tr>
<td>(119)</td>
<td>ApoE−/−mice (male)</td>
<td>60-s cycles of $FiO_2$ 21%−5% for 8h/day for 6 weeks</td>
<td>TNF-α, IL-6,</td>
<td>↑</td>
</tr>
<tr>
<td>(120)</td>
<td>Sprague-Dawley rats (male)</td>
<td>30-s cycles of $FiO_2$ 21% to 7% 6h/day for 5 weeks</td>
<td>Adiponectin</td>
<td>↓</td>
</tr>
<tr>
<td>(121)</td>
<td>Sprague-Dawley rats (male)</td>
<td>90-s cycles of $FiO_2$ 21% to 7% for 8h/day for 7 days</td>
<td>M1 macrophages</td>
<td>↓</td>
</tr>
<tr>
<td>(122)</td>
<td>C57BL/6J mice (male)</td>
<td>60-s cycles of $FiO_2$ 21% to 5% for 12h/day for 6 weeks</td>
<td>CRP</td>
<td>↑</td>
</tr>
</tbody>
</table>

1.1.7.4 Sympathetic Activation

Interruption by hypoxia stimulates sympathetic nervous system activity, leading to vasoconstriction and systemic hypertension. Both baro- and chemoreflexes are involved in sympathetic regulation of blood pressure (123). The baroreflex provides a negative feedback loop in which elevated blood pressure inhibits sympathetic outflow, decreases heart rate, and, thus, lowers blood pressure. In a similar fashion, decreased blood pressure activates baroreflex mechanisms, causing heart rate and, thus, blood pressure to rise. Depressed baroreceptor sensitivity could lead to sympathetic activation. In subjects with sleep apnea, frequent nocturnal spikes in blood pressure can result in downregulation of baroreceptor sensitivity. However, the results of experimental studies are inconsistent, with reports of both decreased and normal sensitivity (124). Induction of OSA in dogs showed a shift of the baroreflex curve to the right, suggesting an elevation in the set point without any sensitivity change (125). On the other hand, acute elimination of OSA by CPAP led to an increase in baroreflex sensitivity and a reduction in the set point in association with decreased blood pressure (126). Therefore, it can be concluded that depression of baroreflex sensitivity may be due to decreased sensitivity or an increased set point. Under chronic hypoxic conditions, carotid bodies also change their hypoxic sensitivity so that there are changes in functional plasticity and long-term facilitation (127). These functional changes are ROS-dependent and may contribute to the persistent reflex activation of sympathetic nerve activity in sleep apnea (128, 129). Exposure of mice to 14-35 days of intermittent hypoxia caused increased production of catecholamines and a significant elevation of blood pressure (130). Adrenal
demedullation and chemical denervation of the peripheral sympathetic nervous system or surgical denervation of peripheral chemoreceptors prevented intermittent hypoxia-induced hypertension in rats (131). Since prolonged elevation of sympathetic nerve activity occurs after short-term exposure to hypoxia, it has been hypothesized that intermittent hypoxia facilitates sympathetic responsiveness to subsequent bouts of hypoxia (132, 133). Sympathetic hyperactivity leads to activation of vascular smooth muscle, vasoconstriction, and elevated blood pressure. Other effects of sympathetic hyperactivity include upregulation of the renin-angiotensin-aldosterone system with a further increase in systemic blood pressure (134). Accordingly, increased levels of peripheral nerve activity, plasma norepinephrine, and urinary catecholamines have been reported in different studies of subjects with OSA (135, 136). A randomized trial of 4 weeks using CPAP reduced excretion of urinary normetanephrine and mean arterial ambulatory blood pressure while it significantly improved baroreflex sensitivity (137). Sympathomodulatory effects of CPAP and O₂ therapy in OSA subjects have been confirmed in several studies (138–141).

1.1.7.5 Endothelial Dysfunction in OSA

Diminished endothelial function is an important consequence of OSA and is frequently measured as impaired endothelium dependent vasodilatation (142). Eventually endothelial dysfunction leads to atherosclerosis, a condition where artery walls become narrow due to the buildup of fatty materials, cholesterol, macrophages, cellular debris, and other substances. These changes create significant reductions in blood flow through the affected artery (143). Although the etiology of atherosclerosis is unknown, several factors such as elevated levels of LDL, low levels of HDL, hypertension, diabetes mellitus, male
gender, obesity, family history, infectious disease, and environmental factors are implicated. Many of these factors lead to endothelial dysfunction and atherosclerosis through a unifying mechanism of oxidative stress and inflammation (144). Various studies show lower levels of circulating NO in OSA, for example, by the reduced levels of serum nitrite/nitrate (by-products of normal NO metabolism) in OSA subjects (145, 146). Many mechanisms have been suggested for endothelial dysfunction due to OSA or IH including (1) interaction on NO and ROS forming peroxynitrite, (2) uncoupling of eNOS, and (3) decreased endothelial expression of eNOS and increased levels of endogenous eNOS inhibitors (88). Due to its short half-life and large volume of distribution, peroxynitrite is hard to measure and these factors explain the lack of difference in nitrotyrosine levels between OSA and healthy subjects (147, 148). However, Jelic and Le Jemtel found an increased expression of nitrotyrosine in endothelial cells derived from OSA subjects (94).

In all the forms of nitric oxide synthase, including the endothelial one, enzymatic activity requires five cofactor groups to incorporate oxygen into the amino acid L-arginine to produce NO. Those cofactors are flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, tetrahydrobiopterin (BH₄), and Ca²⁺-calmodulin. If nitric oxide synthase lacks L-arginine or another of the necessary cofactors, it will produce superoxide anion instead of NO through an uncoupled state of nitric oxide synthase (149). Antoniades et al. reported that increased ROS production during hypoxia could lead to BH₄ oxidation and increased levels of arginase II that degrades L-arginine, leading to further eNOS uncoupling (150). Subjects with OSA have increased levels of asymmetrical dimethylarginine (ADMA), a competitive inhibitor of NOS (151). Studies by Tanaka et al. suggest that eNOS activation is sensitive to
regulation by redox status and that oxidative stress leads to decreased eNOS phosphorylation, so reducing its activity (152), while Jelic and Le Jemtel supported the latter findings when they reported decreased ratios of total phosphorylated eNOS in endothelial cells from OSA (94). Figure 1-5 explains how OSA can lead to atherosclerosis through oxidative stress and inflammatory mediated mechanisms.

Figure 1-5: OSA/IH can lead to oxidative stress, which through many mechanisms can cause endothelial function, which eventually progresses to atherosclerosis.

1.1.8 Antioxidant use in OSA

Although the link between OSA and oxidative stress is well established, there are not many studies exploring the effects of antioxidant treatment on oxidative stress markers and physiological parameters in OSA subjects. Sadasivm et al. treated 10 OSA subjects with oral N-acetylcysteine (NAC – acts as GSH) 1800mg daily for thirty days and reported improvements in Epworth Sleepiness Scale score, AHI and oxygen desaturation index (ODI), decreased plasma levels of lipid peroxidation and increased levels of reduced glutathione (153). Wu et al. randomized 40 OSA subjects into two groups; treated with 1500mg carbocysteine daily or CPAP for six weeks. They reported improvements in Epworth Sleepiness Scale score, AHI, ODI, and oxygen saturation levels to an extent similar to the CPAP treated group. Treatment with carbocysteine also decreased plasma MDA levels, increased plasma SOD and nitric oxide levels, and decreased intima media thickness. However, there was no change in flow-mediated dilation (FMD) at the end of treatment (154). Grebe et al. assessed FMD after 0.5g intravenous injection of Vitamin C in 10 age matched OSA subjects and reported significant increases in FMD to levels comparable to the control group.

In animal models of OSA (Table 1-3), several antioxidant therapies prevent adverse biological outcomes of CIH. However, most of these studies were performed on a small
sample size and for short duration of time. Moreover, antioxidant treatment can be ineffective and, in some cases, can instead be harmful due to pro-oxidant effects (155). These findings provide promising use of antioxidants OSA subjects that warrants further investigation.
Table 1-3: Outcomes of antioxidants treatment in different rodent models of disease

<table>
<thead>
<tr>
<th>Reference</th>
<th>Species</th>
<th>Antioxidant</th>
<th>Dose and duration</th>
<th>Outcome</th>
</tr>
</thead>
</table>
| (156)     | Balb-C mice   | N-acetylcysteine | 10mg/kg for 14 days i.p. | - ↓ activation of NF-κB  
|           | (male)        |                  |                   | - ↓ expression of TNF- α and iNOS  
|           |               |                  |                   | - ↓ expression of Caspase-3 and -6 |
| (157)     | Wistar rats   | N-acetylcysteine | Subcutaneously 300mg/kg for 6 weeks | - ↓ IH mediated Beta cell apoptosis  
| (male)    |               |                  |                   | - ↑ Bcl2 and ↓ Bax |
| (158)     | Sprague Dawley rat | N-acetylcysteine | Oral gavage 300mg/kg daily | - ↓ apoptosis in penile tissue  
| (male)    |               |                  |                   | - ↓ endoplasmic reticulum stress proapoptotic factors  
|           |               |                  |                   | - Improved erectile function |
| (159)     | Wistar rat    | Tempol           | 100mg/kg i.p. before CIH and on day 28 | - ↓ BP  
| (male)    |               |                  |                   | - ↓ plasma levels of norepinephrine, epinephrine and MDA |
| (160)     | FVB mice      | Apocynin SOD MnTMPyP | 3mg/kg daily i.p.  
| (male)    |               |                  | 5mg/kg daily i.p. for 4 weeks | - ↓ myocardial VCAM-1 expression  
<p>|           |               |                  |                   | - ↓ myocardial MDA, apoptosis and fibrosis markers |</p>
<table>
<thead>
<tr>
<th>Study Ref.</th>
<th>Species</th>
<th>Treatment</th>
<th>Route of Administration</th>
<th>Outcomes</th>
</tr>
</thead>
</table>
| (161)     | Sprague Dawley rat (male) | Pentoxyfilline | Oral gavage              | - ➤ plasma antioxidant capacity  
- ➣ plasma MDA levels  
- ➤ epididymal sperm parameters  
- ➤ activity of SOD, catalase and glutathione in testis |
| (162)     | Sprague Dawley rat (male) | Allopurinol   | Oral gavage              | - ➤ ACh-mediated vasodilation in gracilis arteries  
- No effects on plasma oxidative stress markers and vessel morphometry |
| (163)     | C57BL/6J mice (male)    | Pitavastatin  | Oral gavage              | - ➣ levels of 4-HNE, TNF-α and superoxide and apoptosis in the myocardium  
- ➣ hypertrophy of cardiomyocytes and perivascular fibrosis |

4-HNE: 4-hydroxynonenal, Bax: bcl-2-like protein 4, Bcl2: B-cell lymphoma 1, IH: intermittent hypoxia, iNOS: inducible nitric oxide synthase, i.p.: intraperitoneal, MDA: malondialdehyde, NF-κB: nuclear factor kappa B, SOD MnTMPyP: superoxide dismutase mimetic manganese (III) tetrakis (1-methyl-4-pyrydyl) porphyrin, TNF-α: tumor necrosis factor-α, VCAM-1: vascular cell adhesion molecule 1
1.1.8.1 Alpha lipoic acid: a potent antioxidant with anti-inflammatory properties

Lipoic acid (LA) or α-lipoic acid (ALA) is a naturally occurring compound that is also known as 1,2-dithiolane-3-pentanoic acid or thioctic acid. It is synthesized enzymatically in plant and animal mitochondria from octanoic acid and cysteine (as a sulfur source). ALA acts as a cofactor for pyruvate dehydrogenase and α-keto-glutarate dehydrogenase activity (164), and is also required for the oxidative decarboxylation of pyruvate to acetyl-CoA, a critical step bridging glycolysis and the citric acid cycle (165). The presence of an asymmetric carbon produces two optical isomers R-LA and S-LA. Only the naturally occurring R isomer is bound to protein and acts as an essential cofactor in biological systems (165). However, synthetic LA is a racemic mixture of R and S isoforms, where S-LA can prevent the polymerization of R-LA to enhance its bioavailability (166). In cells containing mitochondria, ALA is reduced in an NADH-dependent reaction with lipoamide dehydrogenase to form dihydrolipoic acid (DHLA) (Figure 6), whereas in cells that lack mitochondria, ALA can instead be reduced to DHLA via NADPH with glutathione (GSH) and thioredoxin reductases (167).

Figure 1-6: Structure of alpha lipoic acid and dihydrolipoic acid

(Original figure)
Unlike GSH, for which only the reduced form is an antioxidant, both the oxidized and reduced forms of LA are powerful antioxidants whose functions include: i) Quenching of reactive oxygen species (ROS) (168); common antioxidants are either water-soluble or lipid soluble agents unlike LA that has both hydrophilic and hydrophobic properties. Being both water and fat-soluble means that ALA is widely distributed in plants and animals in both cellular membranes and in the cytosol (169). Therefore, it can elicit its antioxidant action in both the cytosol and plasma membrane in contrast to vitamin C (which is lipophobic) and vitamin E (which is lipophilic). ii) Regeneration of exogenous and endogenous antioxidants such as vitamins C and E, and GSH, iii) chelation of metal ions: because of the presence of two thiol groups, LA and DHLA both have metal chelating properties. In fact, ALA is a potent chelator of divalent metal ions in vitro and forms stable complexes with Mn$^{2+}$, Cu$^{2+}$, Fe$^{2+}$, Pb$^{2+}$, and Zn$^{2+}$ (170). iv) Reparation of oxidized proteins, v) induction of antioxidant gene transcription: normally, Nrf2 is located in the cytoplasm and kept dormant by a cytoplasmic repressor Kelch-like ECH-associated protein 1 (Keap1). A variety of activators release and translocate Nrf2 into the nucleus, where it can regulate the expression of antioxidant enzymes (171). LA is one such inducer of Nrf2-mediated antioxidant gene expression (166). vi) Inhibition of the activation of NF-κB (172, 173): The NF-κB protein complex is normally located in the cytoplasm in an inactive form by virtue of its binding to a family of inhibitor of NF-κB (IκB) proteins. Upon cell stimulation by a wide variety of stimuli, signal responsive IKK α and β (TNF-α-inducible IκB kinase complex also known as IKK1 and IKK2) are activated, resulting in the phosphorylation of IκB and its proteasomal degradation. IκB degradation liberates NF-κB, allowing it to translocate to the nucleus and induce gene
expression (174, 175). It is been shown that LA inhibits IκB degradation and NF-κB-dependent gene expression by inhibition of IKK2, suggesting that LA inhibits NF-κB activation independent of its antioxidant function (176). Antioxidant and anti-inflammatory mechanisms of LA is depicted in Figure 1-7. This impressive array of cellular and molecular functions has raised considerable interest for the use of this substance as a nutritive supplement and also as a therapeutic agent in clinical studies, especially in diseases promoted by oxidative stress and inflammation (Table 1-4) including diabetes.

In summary, OSA is a chronic disease affecting millions of people worldwide and intermittent hypoxia that can lead to CVD through oxidative stress and inflammation. Although treatment with CPAP can ameliorate symptoms and adverse outcomes of OSA on the short run, the long-term effects of CPAP on CVD is questionable especially given poor patient adherence. This necessitates looking into other options for treatment if not OSA at least its underlying mechanisms of CVD. Antioxidant therapy seems promising and should be considered as adjunctive therapy in OSA, especially ALA.
Figure 1-7: Antioxidant and anti-inflammatory mechanisms of alpha lipoic acid.

### Table 1-4: Selected clinical trials with ALA treatment and outcomes

<table>
<thead>
<tr>
<th>Reference</th>
<th>Method</th>
<th>Dose and duration</th>
<th>Measured parameters</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>(177)</td>
<td>360 obese subjects with T2DM, HT, or hypercholesterolemia randomized to: (a) ALA 1200 mg/day (b) ALA 1800 mg/day (c) Placebo</td>
<td>20 weeks</td>
<td>– BW, waist circumference, body fat, BP, FBS, TC, LDL, HDL, TG</td>
<td>– ALA 1800 mg/day led to a modest weight loss in obese subjects</td>
</tr>
<tr>
<td>(178)</td>
<td>59 subjects with T2DM in three groups: (a) PL (n = 19; 10 min, bid) (b) QALA (n = 20; 60 mg CoQ10 + 100 mg ALA + 200 mg Vit E) (c) PL + QALA</td>
<td>3 months</td>
<td>– CRP, LDH, MDA – Serum antioxidant level (CoQ10, α and τ-tocopherol, β-carotene) - Echocardiographic parameters of left ventricular function</td>
<td>– Combined therapy had better results in increasing antioxidant levels, decreasing MDA and improving left ventricular function</td>
</tr>
<tr>
<td>(179)</td>
<td>30 T2DM subjects (a) ALA (600 mg/day, iv) (b) Placebo</td>
<td>21 days</td>
<td>– Blood levels of ADMA (NOS inhibitor).</td>
<td>– ALA decreased plasma levels of ADMA.</td>
</tr>
<tr>
<td>(180)</td>
<td>14 T2DM subjects (a) ALA (400 mg/day)</td>
<td>4 weeks</td>
<td>– Markers of oxidative stress – BP</td>
<td>– ALA decreased markers of oxidative stress and HDL, had a</td>
</tr>
</tbody>
</table>
| (b) Placebo | - Lipid profile, CRP | borderline effect on BP ($p = 0.06$) and LDL ($p = 0.07$)  
- No significant effect on CRP, TC, and TG |
| (181) | 22 obese subjects with IGT |
| (a) ALA (600mg/day, iv, n=13) | 2 weeks | - LDL, ox-LDL, VLDL, TG, TC, FFAs, insulin sensitivity index  
- MDA, 8-isoprostone  
- TNF-$\alpha$, IL-6, plasma $\alpha$-tocopherol |
| (b) Placebo (n = 9) | - improved insulin sensitivity index  
- Decreased LDL, ox-LDL, VLDL, TG, TC, FFAs, MDA, 8-isoprostone, TNF-$\alpha$, IL-6 |

1.2 OSA and pregnancy

Sleep and pregnancy are two unique states that involve extensive dynamic physiological changes, and both states can impact respiration with systemic consequences (182). SDB is common in pregnant women and manifests as increased resistance of the upper airway, snoring, and OSA (182). Although snoring is the most common form of SDB in pregnant women, OSA is considered to be the most severe form due to IH and sleep fragmentation that worsens as pregnancy progresses (183). Furthermore, the unprecedented increase in obesity prevalence worldwide in women of reproductive age increases the risk of developing OSA (184). In addition, SDB increases adverse pregnancy outcomes including gestational hypertensive disorders (GHT) and gestational diabetes mellitus (GDM) (183). Thus, appropriate management and timing of diagnosis is important for improving maternal and fetal outcomes.

1.2.1 Prevalence

The prevalence of snoring in pregnant women in the third trimester ranges between 11.9% and 49% as reported by cross-sectional studies (185–187). Longitudinal studies report that snoring increases from 8% to 21% from the first to the third trimester, respectively (188, 189). Pregnant women also report increased frequency of daytime sleepiness, gasping, choking and breathing pauses throughout pregnancy (190). Due to the lack of large longitudinal cohort studies using PSG and the reliance on reported questionnaires that lack objective validation, the prevalence of OSA in pregnancy remains largely unknown. Moreover, the upper airway is significantly narrowed in the third trimester
when compared to post-delivery, indicating that diagnosis of OSA might be transient with no long-term pathological outcomes such as CIH. Nonetheless, it is important to consider the short-term maternal impact of OSA (191).

Only one recent prospective cohort study assessed AHI in 3705 nulliparous women who underwent in-home sleep disordered breathing assessment in early (6-15 weeks) and mid-pregnancy (22-31 weeks). The corresponding prevalence of OSA (AHI>5) in early pregnancy was 3.6% and 8.3% in mid-pregnancy (192). A small cohort study of 105 women who underwent PSG in the first and third trimester showed prevalence of 10.5% and 26.7%, respectively. However, most of the participants were either overweight or obese. After considering obesity as a confounding factor, the prevalence was reduced to 8.4% in the first and 19.7% in the third trimester (193).

### 1.2.2 Risk factors of pregnancy and OSA

Several hormonal, cardiovascular, and mechanical changes can lead to or even exacerbate pre-existing OSA during pregnancy (183). For instance, increased estrogen levels during pregnancy can induce nasopharngeal mucosal edema and cause narrowing and lead to resistance to airflow (194). It is plausible that estrogen-mediated changes cause an increased risk of SDB in pregnant women since nocturnal nasal congestion is an independent risk factor for SDB in the general population. Moreover, more than 42% of pregnant women suffer from pregnancy rhinitis in the third trimester, which can lead to increased nasopharyngeal resistance (194). Increased maternal blood volume can contribute to fluid displacement that can affect upper airway patency during sleep (194). An elevated
diaphragm during pregnancy can reduce functional residual capacity by 20-25%, which will decrease maternal oxygenation and decrease caudal traction on the trachea and pharynx, enhancing the collapsibility of the pharynx (195). Finally, frequent awakenings can lead to sleep deprivation, which then impairs airway muscle activity and cause upper airway collapsibility (196). Obesity and age are risk factors for OSA not only in the general population, but also in pregnant women (193, 197). Women that are obese and overweight to pregnancy are more vulnerable to OSA (198, 199). However, one study reported that weight gain during gestation was not associated with third trimester OSA (193), possibly because fat deposition in some locations (e.g. soft tissue in neck region) could have a greater influence on developing new onset OSA rather than total weight gain (186). Other risk factors for OSA in pregnancy include: upper airway abnormalities (197), chronic hypertension (200, 201), and smoking (195).

1.2.3 Potential pathological mechanisms in pregnancy and OSA

As discussed earlier, OSA is characterized by intermittent hypoxia and sleep fragmentation, resulting in oxidative stress and inflammation in OSA subjects that are associated with several diseases including preeclampsia, gestational diabetes, miscarriage, fetal growth restriction and preterm labor (202, 203). OSA also increases sympathetic activation that continues in the daytime and is in part responsible for increased blood pressure (204). Pregnant women with preeclampsia have elevated blood pressure. OSA in pregnancy increases the risk of developing preeclampsia or exacerbate it (182). OSA can lead to pancreatic cell dysfunction and insulin resistance that can alter glucose metabolism and
put pregnant women at risk of GDM (205). Potential mechanisms involved in OSA and maternal/fetal outcomes are shown in Figure 1-8.

**Figure 1-8: Potential mechanisms involved in maternal/fetal complications of pregnancy-induced OSA**

(Original figure)
1.2.4 OSA and adverse maternal outcomes

1.2.4.1 Gestational diabetes mellitus

GDM is defined as glucose intolerance with onset during some pregnancies, which is normalized post-delivery (206). The prevalence of GDM can reach 7% in pregnancies and increases with maternal obesity (182, 207). GDM is associated with many adverse maternal and fetal outcomes ranging from spontaneous abortions and congenital anomalies in the first trimester to excessive fetal growth and stillbirth in the third trimester (206). Furthermore, GDM is associated with at least ten complications if left untreated, according to the International Association of the Diabetes and Pregnancy Study Groups (IADPSG) criteria (206, 208). Several studies show a clear link between GDM and OSA in pregnant women. In a retrospective cohort study done by Spence et al. involving 305,001 women (having 266 cases of OSA) who gave birth between 2008-2014, OSA was associated with a higher odds ratio of GDM: 2.87; 95% CI, 2.16-3.82 after adjusting for demographics, obesity, and other comorbidities associated with OSA (209). Louis et al. examined 57 women with complications in their pregnancies due to OSA for eight years (between 2000-2008) and found that OSA was associated with increased risk of GDM (OR, 4.60; 95% CI, 1.52-13.90) (210). Furthermore, a more recent meta-analysis of eight studies of pregnant women with OSA also concluded that OSA is associated with increased risk of GDM (OR, 1.71; 95% CI, (1.23, 2.38) (211).
1.2.4.2 Hypertensive disorders

Hypertensive disorders occur in about 5-10% of pregnancies including preeclampsia (PE), eclampsia, chronic hypertension, and gestational hypertension (GHT) (182). GHT can be defined as a newly diagnosed systolic blood pressure of >140 mmHg or a diastolic blood pressure of >90 mmHg after 22 weeks of pregnancy. PE occurs when proteinuria or signs and symptoms of target organ damage due to vasoconstriction and endothelial activation develops (212). PE is characterized by an impaired invasion of fetal trophoblasts. This inhibits remodelling of maternal spiral arteries that eventually leads to the impedance of blood flow to the placenta that reduces the amounts of oxygen and nutrients delivered to the fetus. A hypertensive state in the pregnant mother increases blood flow to the placenta towards the end of the second or third trimester of gestation (213). Complications subside after delivery, suggesting that PE is a condition that originates from the placenta (214). PE is associated with significant morbidity and mortality in both mother and baby (215).

PE has similar risk factors to OSA, such as obesity and maternal age, which makes it difficult to examine the association between these two conditions. However, OSA can contribute to PE through placental hypoxia, oxidative stress, inflammation and endothelial dysfunction, while PE can worsen OSA via increasing upper way edema (182). Chen et al. performed a large cohort study that included 791 women with PSG-diagnosed OSA prior to pregnancy, and found that these women has an increased risk of developing preeclampsia (OR, 1.6; 95% CI (2.16-11.26) (216). Facco et al. examined 3,306 women with AHI>5 and reported that hypertensive disorders of pregnancy occurred in 433 of the women (13.1%), and that PE occurred in 199 (6.0%). They also found a statistically significant association
between AHI>5 during pregnancy and PE (adjusted OR, 1.94; 95% CI 1.18-3.23) (192). Furthermore, a meta-analysis of nine studies shows an increased risk of preeclampsia in women with OSA (OR, 2.63; 95% CI (1.87-3.70) (211). In summary, several studies support an association of OSA with hypertensive disorders during pregnancy.

1.2.4.3 Maternal morbidity, miscarriage and preterm birth

Despite improved quality of treatment, increasing rates of severe maternal morbidity and mortality continue (217). Louis et al. analyzed a sample of 55,781,965 pregnancy-related hospital discharges from 1988-2009 to determine length of hospital stay, in-hospital mortality, and hospital discharge codes, to identify OSA and other outcomes. The prevalence of OSA was 3/10,000; however, this increased dramatically from 0.7% in 1998 to 7.3% in 2009, representing an annual increase of 23%. OSA was associated with increased odds of eclampsia (OR, 5.4: 95% CI (3.3-8.9), pulmonary embolism (OR, 4.5: 95% CI (2.3-8.9), and cardiomyopathy (OR, 9.0: 95% CI (7.5-10.9), even after adjusting for confounding factors such as obesity. There was a five-fold increase in odds of in-hospital mortality in women with OSA. Obesity exacerbated the adverse effects of OSA on the outcomes discussed earlier (207). Bourjeily et al. found a 0.12% rate of OSA in pregnant women from a sample size of 1,577,632 collected from The National Perinatal Information Center in the US between 2010-2014. They also reported a 2.5-3.5-fold increased risk of severe complications such as cardiomyopathy, congestive heart failure and hysterectomy. Moreover, the odds of admission to intensive care were higher (OR, 2.74; 95% CI (2.36-3.18) and significantly longer hospital stays were prescribed (5.1 + 5.6 vs. 3.0 + 3.0 days, $P>0.001$) (218).
Overweight and obese women with OSA are more likely to have had a miscarriage compared to women without OSA according to a retrospective study (219). However, in this study, the age of onset of OSA and timing of miscarriage were unknown. According to Louis et al., OSA is also associated with preterm birth after adjusting for confounding factors (aOR, 2.6: 95% CI (1.02-6.6) (210). Moreover, a meta-analysis by Li et al. showed an increased risk of premature delivery in women with OSA (OR, 1.47; 95% CI (1.14, 1.91) (211).

1.2.5 OSA and adverse fetal outcomes

Studies that examined the effect of OSA on birth weight have produced conflicting results. A retrospective cohort found no difference in small-for-gestational age babies in women with PSG-confirmed OSA, when compared with obese and non-obese controls (207). Other studies also suggest no association between OSA and fetal growth and prematurity (220, 221). Moreover, a recent meta-analysis reported no significant difference in birth weight when comparing pregnant women with and without OSA. Contrary to these findings, Fung et al. reported impaired fetal growth in 43% of OSA cases vs. 11% of controls (OR, 6; (95% CI (1.2-29.7) (222). Pamidi et al. also found an association between OSA and low birth weight (OR, 1.39; 95% CI (1.14-1.65) (223). The contradictory findings of these studies on OSA and birth weight can stem from confounding factors such as obesity and diabetes. It is well documented that increasing BMI and gestational diabetes are associated with excessive fetal size (224). Thus, the low number of women with small-for-gestational-age infants will underpower studies. There is no evidence that fetal heart rate abnormalities in offspring of pregnant women with OSA (220).
Management of OSA during pregnancy

Some behavioral strategies for improving OSA in pregnancy include weight gain control, left lateral or elevated head of bed sleeping positions, avoiding smoking, alcohol, and caffeine, treating nasal congestion, and regular exercise (225). Women with predisposing risk factors for developing OSA are recommended to achieve normal weight both before pregnancy and postpartum (225). Zaremba et al. found that raising the upper body during sleep reduced AHI from 7.7 ± 2.2/h in non-elevated to 4.5 ± 1.4/h in 45° elevated upper body position (p = 0.031) in women during the 48 hours after delivery (226). There are no pharmacological treatments for OSA in pregnancy or in general.

The most common non-pharmacological treatment in OSA for the general population is CPAP. It is generally safe and well tolerated during pregnancy and adherence can reach 50-60% (227). Several studies show improvements in maternal and fetal outcomes for women with OSA, such as lowering blood pressure and total peripheral arterial resistance and greater fetal movements (228, 229). For example, Edwards et al. show that eleven pregnant women with PE and who underwent PSG had significant blood pressure reductions during a single night use of CPAP (129 ± 4 over 73 ± 3 mmHg) compared to a control night without CPAP (149 ± 6 over 93 ±5 mmHg) (228). These studies had small sample sizes and suggest that large-scale studies are needed to better evaluate the benefits of CPAP on pregnancy outcomes as well as more effective monitoring of CPAP use.
1.2.7 Pregnancy in patients with pre-existing OSA

There are no studies examining maternal or fetal outcomes in pregnant women previously diagnosed with OSA. Given the evidence that OSA during pregnancy is associated with adverse pregnancy outcomes, screening for women at risk for developing OSA is crucial before pregnancy. In addition to obesity, increased age, hypertension, and snoring are also associated with OSA. These predisposing risk factors may be reliable indicators for pre-existing OSA in early pregnancy (182). Women with pre-existing OSA should also be assessed multiple times throughout their pregnancy since the severity of OSA can vary throughout pre-pregnancy, during pregnancy, and the postpartum period (225). For instance, symptoms worsen with increased nasal congestion and weight gain (229). Furthermore, clinical and experimental studies examining the effects of OSA/intermittent hypoxia on placental circulation, development, and function throughout pregnancy are needed to better understand how OSA could lead to adverse maternal and fetal outcomes that originate from the placenta (e.g. PE and intrauterine growth retardation (IUGR)).

1.3 Developmental programming and cardiometabolic disease

The Developmental Origins of Health and Disease (DOHaD) hypothesis by Barker in 1985 (230) predicts that stress during critical periods of embryonic and fetal development can trigger adaptive structural, physiological that result in permanent adverse consequences to life-long health. We now know that manipulating fetal nutrition and/or oxygenation can also result in fetal programming and disease later in life (231–233). Thus, chronic conditions such as obesity, diabetes, maternal excess/deficit nutrition, environmental exposures and
hypoxia, can influence placental and fetal epigenome programming, inducing cardiometabolic disease in adulthood and transmission of heritable risk to the next generation (234). Low birth weight, a hallmark of poor fetal growth, followed by growth catch-up, is linked to an increased risk for adult metabolic syndrome and CVD (235–239). Although epidemiological studies present a clear association between low birth weight and CVD, much of our recent knowledge is derived from animal models that provide insight into the mechanisms by which IUGR can program increases cardiovascular risk in later life (240–243). It is important to mention that sex differences in response to fetal insults have been documented in several animal models of developmental programming with phenotypic outcome linked to the severity of the insult (244).

### 1.3.1 Impaired fetal growth and later cardiovascular disease in life

Low birth weight can result from impaired growth in utero (245), preterm birth (246) or a combination of both. It is defined as birth weight below 2.5kg in humans, irrespective of gestational age (247). IUGR is characterized by small for gestational age infants with a birth length and/or weight below the 10\(^{th}\) percentile for gestational age (248). It is a result of nutrient and/or oxygen deprivation to the fetus due to environmental or genetic factors (249). Several epidemiological studies propose a link between low birth weight with not only with long-term CVD, but also with other diseases such as metabolic syndrome (250), insulin resistance (251) and kidney disease (252). An earlier study by Barker et al., reported that the incidence of ischemic heart disease was higher in men (n=10,141) with low birth weights (253). Since these findings, many other studies have
confirmed these observations (238, 254–257). A retrospective study published by Rich-Edwards et al. involving 121,700 women who were monitored since 1976, revealed a strong association between full-term low birth weight (<2268g) and risk for CVD (RR, 1.45; 95% CI (1.05 - 2.10) despite adjustment for confounding factors such as socioeconomic status and adult lifestyle (238). The association between low birth weight and CVD is stronger when there is accelerated growth after birth (255, 258–260). Most of these studies suggest that small for gestational age and not low birth weight leads to CVD during adulthood.

The catch-up growth hypothesis was introduced by Lucas and Singhal and proposes that low birth weights due to adverse gestational conditions will lead to accelerated growth later in life, which can predispose offspring to cardiometabolic disease during adulthood (261, 262). Several studies report that postnatal weight catch-up in low birth weight individuals leads to increased blood pressure (259), impaired cognitive function (263), obesity (264), type II diabetes (265) and CVD (266). For example, Fagerberg et al. obtained the medical records of 58-year-old men and their weights at birth and also at 18 years of age. Metabolic syndrome was more prevalent in men who were born with low weight and there was accelerated catch-up growth until they were 18 years of age (267). A recent systematic review highlights the implications of low birth weight on CVD later in life. More precisely, catch-up growth in low birth infants may be a more significant factor in developing CVD and its risk factors than low birth weight alone (268).

1.3.2 Animal models of IUGR

To better understand the short- and long-term consequences of IUGR, several animal models using dietary manipulation and/or placental insufficiency have been developed
(269). Table 1-5 highlights some of the techniques used in different species. However, differences in outcomes can arise from variations in species, strains, timing and the magnitude of intervention. Species-specific factors such as litter number, placentation and gestation length, should also be considered before choosing a specific animal model (269). Using mice models for example is appealing as they provide a variety of genetic IUGR models, are easy to maintain, relatively inexpensive, and have short gestation time. However, the surgical intervention of mice and imaging of their placentas and fetuses can be technically challenging.
## Table 1-5: Selected animal models of intrauterine growth retardation (IUGR)

<table>
<thead>
<tr>
<th>Reference</th>
<th>Species</th>
<th>Intervention</th>
<th>Characteristics</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>(270–272)</td>
<td>Mouse</td>
<td>Erk -/-</td>
<td>Fetal growth restriction with 25–40% reduction of visceral organ growth</td>
<td>- Small size and social nature thus easy to maintain and relatively inexpensive to house</td>
<td>- Small size means may be problematic to manipulate surgically</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>40% die at birth from acute respiratory failure, similar to respiratory distress syndrome in humans</td>
<td>- Short gestation reduces the time and expense especially to second and third generation studies</td>
<td>- Imaging of the fetus or placenta can be technically challenging</td>
</tr>
<tr>
<td></td>
<td></td>
<td>eNOS-/-</td>
<td>Fetal growth restriction with brain sparing, hypoxia and reduced placental system A transport</td>
<td>- Can be difficult to follow serially postnatally, due to cannibalization and the challenge of marking newborn mice</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Protein restriction</td>
<td>Fetal growth restriction with adiposity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(273–277)</td>
<td>Rat</td>
<td>Uterine artery ligation</td>
<td>40% fetal growth restriction with brain sparing high level fetal loss and resorption</td>
<td>- Useful for intergeneration studies especially cognitive</td>
<td>- Altricial young</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Fetal growth restriction with brain sparing fetal mortality 14%</td>
<td>- Large enough for complex surgical intervention</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Uterine artery occlusion (60 min)</td>
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<td></td>
<td>Short gestation, large litters</td>
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<tr>
<td></td>
<td>L-NAME administration</td>
<td></td>
<td>Fetal growth restriction up to 20%, increased stillbirth dependent on dose regimen</td>
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<tr>
<td></td>
<td>Hypoxia</td>
<td></td>
<td>4–37% fetal growth restriction, varied level of exposure</td>
<td></td>
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<tr>
<td></td>
<td>Protein restriction</td>
<td></td>
<td>15% fetal growth restriction</td>
<td></td>
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</tr>
<tr>
<td>(278, 279)</td>
<td>Guinea pig</td>
<td>Uterine artery ligation</td>
<td>40–60% fetal growth restriction in a proportion of fetuses</td>
<td>- Longer gestation</td>
<td>- Less common laboratory animal so specific</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Extensive trophoblast invasion</td>
<td></td>
</tr>
</tbody>
</table>
| Maternal nutrient restriction | Both acute fasting and chronic restriction effective 10–39% fetal growth restriction with brain sparing altered trophoblast density, placental barrier thickness | - Haemomonochorial placenta reagents/equipment more expensive  
- Longer gestation, larger animal, - smaller litters thus more expensive |
<table>
<thead>
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</tr>
</thead>
<tbody>
<tr>
<td>(280, 281) Sheep Hypoxia</td>
<td>25% fetal growth restriction systolic and diastolic fetal cardiac dysfunction</td>
<td>- Serial sampling from both sides of the placental barrier unanaesthetised</td>
</tr>
</tbody>
</table>
| Nutrient restriction | Mild FGR, 17% reduced uterine blood flow, reduced placental capillary density | - Placentation is not closely similar to human  
- Large animal facility needed |

Sheep conceptus relevant to human fetal physiology
- Consistent gestation with predominantly singleton pregnancies

1.3.3 Mechanisms and consequences of IUGR and developmental programming

Influences during gestation and early life that alter growth and development can lead to long-term cardiovascular consequences later in life and may impact subsequent generations, implicating a role for epigenetic processes (282). Animal studies support the DOHaD hypothesis and provide insight into the mechanisms involved. Several fetal stress factors share some common mechanisms that can lead to cardiometabolic disease, with slight disparities in their relative contribution depending on the stress applied (283). Some mechanisms that have been reported include: (i) reduced nephron numbers and impaired kidney development (284), (ii) vascular alterations, including endothelial dysfunction and vascular remodelling (285), (iii) cardiac alterations, including reduced cardiomyocyte number, fibrosis and hypertrophy, causing cardiac dysfunction (286), (iv) insulin resistance in adipose tissue and skeletal muscle (287, 288), (v) alterations in the renin-angiotensin-aldosterone system (RAAS) (289), and (vi) leptin resistance and hyperphagia (290). A summary of the main mechanisms associated with fetal programming of cardiometabolic disease is shown in Figure 1-9. Animal studies also report a sexual dimorphism in fetal programming of cardiometabolic disease with female offspring exhibiting protective effects (291, 292). Sex steroids are thought to be involved in the sexual dimorphism with estrogen being a protective factor while testosterone being a permissive for disease susceptibility (282). However, it is yet to be established whether women are at lower risk than men for CVD programming and what the roles of sex steroids are in the process.
Figure 1-9: Potential mechanisms involved in developmental programming of cardiometabolic disease.

(Original figure)
1.3.4 Role of hypoxia in developmental programming

Fetal hypoxia is the most common outcome of complicated pregnancies (293). Recent studies reveal a strong link between fetal hypoxia, IUGR, and the developmental programming of cardiometabolic disease (233). In response to acute hypoxia, the fetus redistributes blood flow away from peripheral tissue to maintain adequate blood flow to the brain (294). Should hypoxia persist, the result of blood flow redistribution can lead to asymmetric fetal growth (offspring with normal growth of brain and heart but stunted organ development) (295, 296). Moreover, chronic hypoxia increases total peripheral resistance and increases cardiac overload, resulting in morphological and functional changes in the fetal heart and the development of CVD later in life (233). An early animal model was the chick embryo model, which enabled researchers to study the direct effects of fetal hypoxia independent of maternal and placental influences. Poor fetal growth, impaired cardiac and vascular function, and heightened sympathetic innervation occurred by the end of the incubation period of chick embryos allowed to develop at high altitude (297–299). Oxygen supplementation prevents fetal growth impairment and cardiac remodeling in sea level chick embryos incubated at high attitude (300). Asymmetric growth restriction and cardiac dysfunction has also been studied in sheep (301), rodents (302) and guinea pigs (303). These findings suggest that pregnancy complications characterized by placental insufficiency can detrimentally affect the fetal cardiovascular system.
1.3.4.1 Vascular dysfunction

Vascular dysfunction occurs in the offspring of animals exposed to chronic hypoxia, as shown in the studies by Davidge et al. where mesenteric arteries had a three-fold increase in vasoconstriction in response to endothelin-1 in adult offspring male (but not female) rats (304). Another study from the same group reported that male (but not female) offspring from dams exposed to hypoxia or nutrient restriction showed an age dependent increase in myogenic responses (7 months vs. 4 months old) that was not seen in controls (305). Impaired NO-mediated dilation in small mesenteric, femoral, and middle cerebral arteries has also been reported in male rat offspring exposed to maternal hypoxia (306–308). The thoracic aortas of 16-month-old male rats exposed to gestational hypoxia have atherosclerotic lesions with significant thickening of the intima (309). Adult offspring rats (5 months old) from mothers exposed to hypoxia have increases in aortic proinflammatory cytokine expression and migration of smooth muscle cells into the intima (310). These findings indicate that maternal hypoxia leads to vascular dysfunction in adult offspring in a gender-specific and age-dependent manner, with males being more susceptible.

1.3.4.2 Metabolic syndrome

The prevalence of metabolic syndrome is on the rise posing a significant health crisis worldwide. The metabolic syndrome is a combination of central obesity and insulin resistance plus any two of the following factors: high triglycerides (TG), low HDL, high blood pressure and high fasting blood glucose (311). The prevalence of metabolic syndrome in adults in the USA increased by more than 35% from 1988 to 1994 and from 25.3% to 34.2%
between 2007 and 2012 (312). Evidence from epidemiological and animal studies suggest a strong association between the prenatal environment and the development of metabolic disease later in adulthood (313). However, only a few studies have examined the association between prenatal hypoxia and metabolic disease in offspring. Camm et al. reported that the expression of hepatic p-Akt and muscle Akt2 were reduced in the offspring of a rat model of hypoxic pregnancy. There were also reductions in hepatic Akt1, Akt2 and PKCζ and muscle GLUT4 expression in offspring of maternal hypoxic pregnancies (314). These findings suggest that prenatal hypoxia can adversely affect insulin signaling in adult rat offspring.

Experiments by Vargas et al. examined the effect of late-gestational maternal hypoxia (12% O2) on post weaning appetite, fat deposition, adipose tissue cytokine expression, hypothalamic arcuate nuclei (ARH) response to exogenous leptin, and appetite in male offspring rats. Their results indicate that maternal hypoxia affected the developing ARH leading to hyperphagia and contributing to adult obesity on a control diet (290). Related findings by Davidge et al. reported that adult offspring of hypoxic dams had increased central fat deposition and adipocyte size accompanied by increased plasma levels of triglycerides, leptin, and free fatty acids. They also reported impaired glucose tolerance and insulin resistance in adult offspring rats exposed to maternal hypoxia (315).

1.3.4.3 Perivascular adipose tissue (PVAT) dysfunction

Adipose tissue was long considered to be a reservoir for energy storage and also having some thermoregulatory functions. However, these perceptions changed when it became clear that adipose tissue, specifically central adipose tissue, is a dynamic endocrine
organ that plays an important role in the development of metabolic and cardiovascular disease (316). A protective role of subcutaneous adipose tissue may arise from its actions as a buffering system for excess fat accumulation (317). PVAT surrounds large, small and resistance blood vessels, as well as large veins and skeletal muscle micro vessels (318). PVAT was initially believed to only provide structural support to blood vessels. However, it is now recognized as a unique fat depot that can actively regulate vascular function due to its ability to produce a variety of adipokines, which exert their effects by autocrine (e.g. adipogenesis), paracrine (e.g. VSMC relaxation/contraction) and endocrine actions (e.g. appetite regulation by leptin)(316).

Factors secreted by PVAT are thought to reach the medial and endothelial layers via direct diffusion or through a reticular network of collagenous conduits connecting and enabling soluble molecules to cross between the PVAT-adventitia-media-intima layers (319). PVAT differences from brown adipose tissue (BAT) and white adipose tissue (WAT) was proposed in a study showing absence of PVAT around the aortic and mesenteric arteries of mice with VSMC-specific PPAR-γ deletion (320). However, PVAT phenotypic and functional properties differ between depots, which are thought to have different neuronal innervations and secretory profiles (321).

PVAT has important roles in thermoregulation (320), vascular contractility (322), inflammation (323), VSMC proliferation (324), endothelial function (325), and vascular redox state (326). A well-studied role of PVAT is its anti-contractile activity that was first observed in 1991 by Soltis and Cassis when they reported that PVAT decreased contractile responses to norepinephrine in the rat aorta (327). Several studies later confirmed this
observation in different vascular beds using a variety of vasoconstrictor agonists (322, 326). Factors that mediate the anti-contractility effects of PVAT are called adipocyte-derived relaxing factors (ADRF) or perivascular-derived relaxing factors (PVRF) (328). Figure 1-10, which has been adapted from Li et al., depicts potential PVRF and their mechanisms of action in the vasculature. Adiponectin is an anti-inflammatory, insulin sensitizing, and antioxidant adipocytokine (329). Several studies highlight the importance of adiponectin as a PVRF with anti-contractile activity. In adiponectin-deficient mice, anti-contractile activity was significantly attenuated (330). Moreover, the anti-contractile function can be completely abolished by adiponectin receptor blockage (331). Adiponectin induces vasorelaxation through multiple mechanisms including: (i) hyperpolarization of VSMC (330), (ii) stimulation of NO release from adjacent adipocytes and endothelium (332), (iii) activation of AMP-activated protein kinase (AMPK) (333), and (iv) increasing the biosynthesis of BH₄ (334). Furthermore, adiponectin can protect against oxidative stress by suppressing NADPH oxidase activity (335). The anti-contractile response is lost in small arteries from humans affected by metabolic syndrome and obesity (331). It has been proposed that obesity or a high fat diet can induce PVAT dysfunction through a triad of hypoxia, inflammation, and oxidative stress (336). Adipocyte hypertrophy causes adipocyte hypoxia, which in turn induces proinflammatory cytokine production triggers NADPH oxidase activity, resulting in a vicious cycle of oxidative stress and inflammation (336). Mice fed a high fat diet for two weeks have increased expression of proinflammatory cytokines and decreased expression of adiponectin (337). PVAT samples obtained from obese subjects (338) and other animal
models of obesity, such as ob/ob (339), db/db (340) and diet induced obese mice (337) have reduced levels of adiponectin.

**Figure 1-10: PVAT-derived vasoactive molecules.**

Methyl palmitate produced by PVAT adipocytes (AC) causes vasodilation by opening the K_v channels on VSMC. H_2S is synthesized in PVAT by CSE and induces VSMC hyperpolarization by stimulating KCNQ-type K_v or K_ATP channels. Leptin induces endothelium-dependent vasodilation by stimulating leptin receptor (LepR), which leads to activation of eNOS via a pathway involving AMPK and Akt and to H_2S production. This H_2S functions as an EDHF and activates endothelial small (SK_Ca) and intermediate (IK_Ca) conductance calcium-dependent K^+ channels via autocrine mechanisms. The resulting hyperpolarization of endothelial cells can be transmitted to VSMC by electrical coupling through myoendothelial gap junction (MEGJ). Leptin also causes endothelium-independent vasodilation by inducing VSMC hyperpolarization through unknown mechanisms. NO and H_2O_2 released from PVAT can elicit vasodilation by activating sGC leading to the synthesis of cGMP. Adiponectin released from PVAT AC can be enhanced by stimulation of β_3 adrenoceptors (β3) and by the NO-
cGMP-PKG pathway. Adiponectin exerts multiple vascular effects: it stimulates NO production from PVAT and from endothelial cells and induces VSMC hyperpolarization by activating TRPM4 channels followed by opening BK$_{Ca}$; Ang 1–7 produced by PVAT acts on endothelial Ang 1–7 receptor (Mas; MAS1 receptor) thereby stimulating endothelial NO production. Besides stimulating sGC activity, NO from PVAT and endothelial cells can also induce/potentiate VSMC hyperpolarization through K$_{Ca}$ or BK$_{Ca}$. (Used with permission ©Xia N, Li H. British Journal of Pharmacology. 2017 Oct 1;174(20):3425-42)

There are no studies we could find on the impact of in-utero perturbations on PVAT health in adulthood. In a study by Zabroska et al., female mice were fed a HFD for 12 weeks prior to mating; and subsequently sacrificed male offspring (raised on a regular diet) at week 12 or 24 who were fed a regular control diet. They measured contractile responses to U46619 (Thromboxane A$_2$ receptor agonist) and norepinephrine in PVAT-intact or -denuded mesenteric arteries. Anti-contractile activity was lost at weeks 12 and 24 and restored by AMPK activation (341). In experiments by Wakana et al., female apolipoprotein E-deficient mice were fed a HFD during gestation and lactation, while offspring were fed a high-cholesterol diet from 8 weeks of age. When mice were 20 weeks old, the thoracic PVAT from male offspring of HFD-fed dams had increased tissue levels and mRNA expression of inflammatory cytokines (IL-6, TNF-, MCP-1). Intra-abdominal transplantation of thoracic PVAT from 8-week old offspring of HDF-fed dams alongside the distal abdominal aorta led to a 2-fold increase in atherosclerotic development in recipient apolipoprotein E-deficient mice compared to transplanting the thoracic PVAT from offspring of normal diet-fed dams (342). Further studies are needed to elucidate the epigenetic mechanisms in developmental programming of PVAT dysfunction.
1.3.5 **Role of Oxidative stress in developmental programming**

Oxidative stress occurs in a myriad of diseases, including CVD (80). During pregnancy, physiological production of ROS aids in placentation and is required for the normal progression of embryonic and fetal development (202). However, the embryo and fetus have low antioxidant capacities, making them more susceptible to excess ROS production and so compromising normal growth. Oxidative stress is associated with CVD risk in early life in infants with low birth weights (283). An relationship between lipid peroxidation and cardiometabolic disease has been established in small for gestational age children (343–345).

Evidence for a role of oxidative stress in developmental programming of adult disease comes from studies where pregnant animals exposed to some insults were also treated with antioxidants. For example, Bi et al. reported increased renal 8-isoprostane levels in response to Ang II in adult male offspring sheep of mothers exposed to glucocorticoid, which was mitigated by the administration of ANG (1-7) (346). Chronic maternal treatment with tempol of rats with reduced uterine perfusion normalized blood pressure and reduced markers of oxidative stress in growth restricted male offspring when compared to control (347). In the vasculature, Xiao et al. administered nicotine to pregnant rats and evaluated vascular oxidative damage and dysfunction in 5-month-old adult offspring where there were increases in MDA, superoxide, and nitrotyrosine protein levels in aortic walls. They also reported impaired relaxations to ACh in the aorta from pregnant rats exposed to nicotine, which were restored by apocynin and tempol administration in a dose-dependent manner.
(348). In other studies, Cambonie et al. fed pregnant rats a diet low in protein and tested carotid vascular function in their adult offspring. There were increases in blood pressure, and vasomotor responses to ANG II, while the vasodilation response to sodium nitroprusside was impaired. Treatment of mothers fed a low protein diet with lazaroid (a lipid peroxidation inhibitor) reversed these adverse vascular effects (349). Other studies examining the effects of antioxidants on offspring outcomes in different biological systems are described in Table 1-6.
Table 1-6: Selected experimental studies on antioxidants treatments and outcomes in the offspring

<table>
<thead>
<tr>
<th>Reference</th>
<th>Species</th>
<th>Insult</th>
<th>Duration</th>
<th>Antioxidant</th>
<th>Outcome in offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td>(350)</td>
<td>Sprague-Dawley rat</td>
<td>Nicotine (osmotic pump)</td>
<td>Day 4 of gestation - day 10 after birth</td>
<td>N-acetyl-cysteine (drinking water)</td>
<td>8 months old:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-↓ nicotine induced increase in BP</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-↓ Ang II induced arterial contractions</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-↑ ACh induced relaxation</td>
</tr>
<tr>
<td>(351)</td>
<td>Balb-c mice</td>
<td>Maternal cigarette smoking</td>
<td>Six weeks prior to mating, through gestation and lactation</td>
<td>L-carnitine (drinking water)</td>
<td>Day 1, 20 and 13 weeks old:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-↑ expression of renal development markers glial-cell line-derived neurotrophic factor and fibroblast growth factor 2</td>
</tr>
<tr>
<td>(352)</td>
<td>Wistar rat</td>
<td>No insult</td>
<td>7th day of gestation until end of lactation</td>
<td>Conjugated linoleic acid (dietary)</td>
<td>70 days old:</td>
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<tr>
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<td></td>
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<td></td>
<td>-↓ in parameters of anxiety</td>
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<td></td>
<td>-↓ levels of MDA in the brain (lipid peroxidation)</td>
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<td></td>
<td></td>
<td>-↑ of glutathione concentration in the brain</td>
</tr>
<tr>
<td>(353)</td>
<td>Sprague-Dawley rat</td>
<td>Dexamethasone injection</td>
<td>Day 16 to 22</td>
<td>Resveratrol (drinking water)</td>
<td>4 months old:</td>
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<td>- Prevented increase in BP</td>
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<td></td>
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<td>- ✈ levels of ADMA</td>
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<td></td>
<td></td>
<td></td>
<td>- ✈ mRNA expression of Ren, ACE and Agtr1a</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(354)</th>
<th>Wistar rat</th>
<th>Low protein diet</th>
<th>During gestation and lactation</th>
<th>Resveratrol (orally)</th>
<th>110 days old:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- ✈ MDA levels in serum and liver</td>
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<td></td>
<td></td>
<td></td>
<td>- ↑ liver GPx and SOD</td>
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<td></td>
<td></td>
<td></td>
<td>- ↑ insulin sensitivity</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(355)</th>
<th>C57BL/6J mice</th>
<th>High fat diet</th>
<th>6 weeks before mating, during pregnancy and 15 days of lactation</th>
<th>Ascorbic acid (drinking water)</th>
<th>8 weeks old:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- ✈ synaptic disruption of dendritic spines and loss of spines</td>
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</tbody>
</table>

1.3.6 Hypoxia-mediated oxidative stress

It is well established that hypoxia can trigger oxidative stress. This leads to the hypothesis that programming of cardiometabolic diseases in response to hypoxia could be mediated by oxidative stress. As discussed above, several studies show that antioxidant treatment of mothers exposed to distress during pregnancy produced favorable outcomes in the offspring. Animal studies demonstrate a clear link between gestational hypoxia, oxidative stress, and programming of CVD in fetal and adult offspring. Zhu et al. studied the effects of perinatal hypoxia on the aorta from 21-day-old rats and reported increased ANG II mediated vessel contraction that was blocked by administering losartan, indicating that AT1 receptors played a dominant role in the enhanced vasoconstriction. ANG II-mediated vasoconstriction was also inhibited by apocynin (NOX inhibitor) or tempol (SOD mimetic). Maternal hypoxia also increased superoxide production and reduced SOD expression in their offspring, accompanied by an enhanced NOX4 expression (356). Giussani et al. reported that maternal hypoxia (13% O2) at day six of pregnancy increased fatal heart, vascular oxidative stress, and aortic wall thickening by the end of gestation. They also reported that adult offspring had markedly impaired NO-mediated vasodilation in femoral arteries, and increased myocardial contractility with sympathetic dominance. Maternal vitamin C treatment prevented the cardiovascular dysfunction in both fetal and adult offspring. Of note is that that maternal vitamin C treatment caused endothelial dysfunction in offspring from normoxic pregnancies (306). Several other studies using rat and sheep models show that maternal antioxidant treatment improved birth weight and vascular function in offspring exposed to gestational hypoxia (357–359).
Studies from the Davidge lab examined the effects of perinatal (360) and postnatal (361) resveratrol treatment in rodents exposed to chronic hypoxia in utero. In one study, rat offspring of dams exposed to chronic hypoxia and fed an HFD or HFD with dietary resveratrol for 9 weeks post weaning. At 12 weeks of age, maternal hypoxia impaired metabolic function in male but not female offspring fed an HFD that was improved by resveratrol treatment. Maternal hypoxia also led to reduced recovery from ischemia/reperfusion (I/R) injury in male, and to a lesser extent, in female offspring fed an HFD and increased cardiac oxidative stress in both. Resveratrol improved cardiac recovery and from I/R and attenuated superoxide levels in both male and female offspring (362). This data indicated that maternal and postnatal targeting of oxidative stress has great therapeutic potential in preventing hypoxic developmental programming of cardiometabolic disease. Although results of antioxidant treatments are promising in animal models of IUGR, systematic reviews of randomized control trials using antioxidant treatments have failed to improve pregnancy outcomes such as PE, preterm birth, and fetal growth restriction (363–366). More alarmingly, antioxidant treatment studies demonstrated increased risk of PE and low birth weight (367, 368). Some of the reasons that may contribute to the disparities in results between animal and clinical use of antioxidants is that oxidative damage in laboratory animals seem to be more responsive to dietary antioxidants and most clinical studies do not consider the baseline nutrition status of participants (369).
1.3.7 Epigenetic mechanisms in developmental programming of CVD

Epigenetics refers to heritable changes in the regulation of gene expression unrelated to nucleotide sequence and chromatin organization of the DNA sequence (370). Epigenetic inheritance allows the propagation of gene activity from one generation of cells to another. It also creates a stable memory of cell identity that maintains genomic function after differentiation (371). Unlike genetic information, epigenetic changes are reversible and respond to environmental influences. Epigenetic changes can occur in early development and induce long-term changes in gene expression, leading to pathological diseases later in life. The three main epigenetic markers are DNA methylation, post-translational histone modifications, and noncoding micro-RNAs (372).

DNA methylation occurs at specific dinucleotide sites or at cytosine bases followed by guanosine (CpG islands) (373). Up to 40% of genes contain CpG islands, of which 70% are methylated (374). Methylated CpG islands serve as docking sites where several protein with the ability to oligomerize can bind and cause chromatin condensation which in turn, leads to gene inactivation and silencing (375, 376). Changes in DNA methylation patterns are involved in CVD and other diseases such as cancer (377, 378). Four major histones (H2a, H2b, H3 and H4) form an octamer and that interacts with eukaryotic DNA to create the nucleosome, the fundamental unit of the chromatin (379). Histones undergo a variety of post-translational modifications, which mainly targets the N-terminal tails protruding from the chromatin fiber (380). The most relevant modifications are acetylation, methylation, and phosphorylation, which have been associated with gene expression in addition to other
processes (381, 382). Micro-RNAs (miRNAs) have recently been identified as regulators of gene expression in different biological systems (372). In humans, over 2500 miRNA’s have been discovered and thought to regulate 60% of all human genes (383).

1.3.7.1 Role of hypoxia and oxidative stress

ROS have been implicated in both genetic and epigenetic alterations in biological systems influencing gene expression and cell differentiation (384, 385). Direct interactions of ROS with DNA cause DNA breakage and damage, allowing access to sites for DNA methyltransferases (DNMT) and hence, promoting DNA methylation (386). Conversely, in vitro studies show that 8-OHdG in CpG nucleotides inhibit the methylation of adjacent cytosines and thus reduce the activity of DNMT and changing methylation patterns (387). The challenge of linking oxidative stress during pregnancy to specific epigenetic target sites in the offspring may be due to our limited ability to track ROS-specific signalling during gestation (386). It is now recognized that intrauterine hypoxia and poor nutrition enhance ROS production (283). Experiments by Rexhaj et al. tested the hypothesis that a restrictive diet during pregnancy induces pulmonary vascular dysfunction in offspring mice through epigenetic mechanisms. They found that endothelium-dependant relaxations were impaired in pulmonary arteries and were accompanied alterations in lung DNA methylation. The administration of nitroxide tempol (an antioxidant) to pregnant dams prevented vascular dysfunction and normalized DNA methylation in the offspring, suggesting an important role of oxidative stress in the developmental programing of vascular pulmonary disease (388). In another model of IUGR, pancreatic beta cells from rat fetuses exhibited reduced expression
of Pdx1 (a gene required for pancreatic development and insulin production), due to histone modifications on the Pdx1 promoter (389).

In the developing heart and brain, fetal hypoxia induces promoter hypermethylation of the glucocorticoid receptor (GR) gene and epigenetic repression of the GR in the offspring (390–392). Gonzalez et al. exposed pregnant rats to hypoxia (10.5% O₂) from day 15 to 21 of gestation. Hypoxic-ischemic injury was significantly increased in 10-day old male and female pups of dams exposed to maternal hypoxia. Dexamethasone injection into the right lateral ventricle produced a concentration dependant reduction in hypoxic-ischemic injury in control pups but not in pups of hypoxic dams. Gene and protein expression of GR was decreased in fetal brains and neonatal hippocampus as a result of maternal hypoxia, with increased promoter methylation of GR and decreased binding of transcription factors on exon 17 and 111 in the fetal brain (390). Related experiments by Xiong et al. also reported decreases in GR exons 14, 15, 16 and 17 transcripts and downregulation of GR mRNA in fetal rat heart exposed to maternal hypoxia. They also showed significant increases in CpG methylation at the binding sites and a decrease in the binding of the transcription factor to GR exon 1 promoter. Treatment of newborn pups with 5-aza-2-deoxycytidine, an inhibitor of DNA methylation, reversed hypoxia-induced promoter methylation, restored GR expression and prevented hypoxia-mediated increase in I/R injury of the heart (391). Fetal hearts of pregnant rats exposed to hypoxia have increased methylation of protein kinase C-ε (PKCε – cardioprotective against ischemia-reperfusion injury) promoter a report by Patterson et al.. Treatment with 5-aza-2-deoxycytidine blocked hypoxia-induced increases in methylation and normalized protein kinase C-ε (PKCε) mRNA and protein levels in both
fetuses and adult offspring (393). The experiments by Patterson et al. also showed that treatment of pregnant rats with an antioxidant (N-acetyl-cysteine) inhibited hypoxia-mediated increases in PKCε promoter methylation, restored PKCε mRNA protein levels to normal, and abolished hypoxia induced increase in the susceptibility of hearts to ischemic injury in the offspring. In summary, findings in animal models suggest a direct effect of gestational hypoxia and oxidative stress on epigenetic regulation in the developing fetus and offspring, which potentially could lead to cardiometabolic disease.

1.3.8 OSA and developmental programming

There are very few studies linking maternal OSA to developmental programming of cardiometabolic disease in the offspring. Given the rise of OSA prevalence in the general population and adverse maternal/foetal outcomes linked to OSA and pregnancy, such as PE and low birth weight, OSA may be a potential risk factor for the developmental programming of adult disease. Currently, only one study has examined the effect of GIH on the cardiometabolic outcomes in offspring, where GIH occurred during the late gestational period (day 13-18 in rats) to mimic pregnancy-induced OSA. This study by Khalyfa et al. reported no effect on birth weights but showed metabolic dysfunction in adult male offspring as reflected by increased body weight, adiposity index, dyslipidemia, insulin resistance, and inflammation of visceral adipose white tissue (VWAT). Using an unbiased method for DNA methylated fraction enrichment in mouse VWAT coupled to a microarray platform, Khalyfa et al identified over 1500 deferentially methylated gene regions associated with 693 genes. Another recent study reported that late GIH increases the susceptibility to
neuroinflammation and alters respiratory motor control in neonatal rats (394). Additional studies are needed to elucidate the mechanisms involved in the developmental programming of cardiometabolic disease in the offspring of mothers with pre-existing OSA.
1.4 Rationale and aims

We hypothesized that treating mice exposed to CIH with ALA can improve vascular function by mitigating oxidative stress and inflammation; we also hypothesized that gestational IH (GIH) can impair main uterine artery function and fetal growth during pregnancy in female mice and result in adverse cardiometabolic outcomes in adult offspring. We tested the mentioned hypothesis using the following aims:

1. Exposing male mice to CIH for 8 weeks with or without treatment with dietary ALA. At the end of the experiment, endothelium-dependent relaxation, oxidative stress and inflammatory markers, aortic gene expression of antioxidants and pro-inflammatory cytokines will be assessed.

2. Exposing pregnant mice to GIH for 14 days then evaluating main uterine artery function, plasma markers of oxidative stress, inflammation, angiogenic and anti-angiogenic factor. Placental morphology, hypoxia, oxidative stress and spiral artery remodelling will also be assessed.

3. Evaluating basic metabolic parameters of 16 weeks old offspring exposed to GIH as well as aortic endothelium-dependant relaxation and PVAT anti-contractility effects. Contribution of adiponectin to the anti-contractility effects of PVAT and its promoter methylation levels in PVAT will be also assessed.
Chapter 2: Alpha lipoic acid improves endothelial function and oxidative stress in mice exposed to chronic intermittent hypoxia

2.1 Introduction

Obstructive sleep apnea (OSA) is characterized by repetitive collapse of the pharyngeal airway during sleep, leading to intermittent hypoxia followed by reoxygenation. These changes can activate several pathological pathways such as oxidative stress and inflammation that can lead to endothelial dysfunction and cardiovascular disease (CVD) (395). Indeed, OSA is considered an independent risk factor for CVD, with cardiovascular events occurring three times more frequently in subjects with OSA compared to controls (396). Animals exposed to intermittent hypoxia (model of OSA), also experience cardiovascular and metabolic sequelae including oxidative stress, hypertension, glucose intolerance, and endothelial dysfunction (5).

Continuous positive airway pressure (CPAP) is the standard treatment in subjects with moderate to severe OSA and improves sleepiness and reduces blood pressure (397). However, 46 to 83% of OSA subjects are non-adherent to CPAP (398). Results from the recent SAVE study indicated that CPAP prescription did not prevent cardiovascular events in subjects with moderate to severe OSA and established CVD when compared to those who received usual care alone (62). This may in part be due to the relatively low adherence (less than 4 hours per night) to CPAP use. This strongly suggests that other treatments are needed to improve cardiovascular health in subjects with OSA.

Alpha lipoic acid (ALA) is a naturally occurring dithiol compound enzymatically synthesized from octanoic acid in the mitochondria. ALA and its reduced form dihydrolipoic acid (DHLA)
act as potent antioxidants though various pathways including reduction of oxidized endogenous antioxidants (Vitamin C and glutathione) and modulation signaling pathways for nuclear factor kappa B (NF-κB) and insulin (399). ALA is a commonly used and readily available dietary supplement. ALA improves endothelial function in subjects with type II diabetes (400), and Alzheimer’s disease (401). ALA also reduces oxidative stress and inflammation in numerous animal models of disease including atherosclerosis (402, 403). We hypothesized that dietary ALA ameliorates endothelial dysfunction in mice subjected to chronic intermittent hypoxia (CIH) by attenuating oxidative stress and inflammation, and restoring nitric oxide bioavailability.

2.2 Materials and Methods

2.2.1 Animals and CIH protocol

Experimental protocols were approved by the Animal Care Centre at The University of British Columbia, Canada (Certificate # A16-0291). Adult male C57BL/6 (10 weeks old) mice were purchased from Charles River (Wilmington, MA). Forty mice were divided into four groups of mice subjected to: 1) intermittent air (IARD) and fed regular diet (Research Diets, New Brunswick, NJ), 2) IA and fed diet containing 2g/kg Bio-Enhanced® Na R-Lipoic Acid (ALA, GeroNova Research, Richmond, CA) (IALA), where the dose was determined from previous studies (404) and estimated to be equivalent to 2000 mg/day in humans, 3) intermittent hypoxia (IHRD), and 4) IH and fed diet containing ALA (IHLA). The diet contained 14% protein, 73% carbohydrates and 4% fat (Catalogue#: D10012M). Mice were housed in customized cages with ports spaced evenly to allow for uniform airflow from all
sides. A gas control delivery system regulated the flow of N₂ and compressed air inside the cages. A combination of flow regulators, oxygen sensors and programmable solenoid valves were used to control the fraction of oxygen inspired (FI,O₂), which could be adjusted over a wide range of CIH profiles. During the 12 hours light cycle (mice are nocturnal animals), FI,O₂ was reduced from 21% to 5-6% for 30 seconds using N₂ gas followed by 30 seconds of compressed air, so returning FI,O₂ back to 21%. This was repeated a total of 60 cycles per hour for 8 weeks. A similar protocol was used for the control groups, where only compressed air was delivered (no N₂). We allowed the mice to acclimate to the hypoxic stimulus by first setting the nadir FI,O₂ at 18% and then gradually reduced every two days by 2% until it reached the desired experimental levels of 5-6% to allow the mice to acclimate to the hypoxic stimulus (405).

2.2.2 Biochemical measures

Mice were euthanized according to the University Animal Care Centre (ACC) guidelines using the inhalant anaesthetic isoflurane (5%) at 1-2 L O₂/minute followed by carbon dioxide until animal stopped breathing and left in chamber for at least 5 minutes after turning off the carbon dioxide. Fasting blood glucose was measured using a glucometer after puncturing the saphenous vein, plasma insulin (Alpco, Boston, MA), tumor necrosis factor-alpha (TNF-α) (R&D systems, Minneapolis, MI) and urinary 8-hydroxy-2’-deoxyguanosine (8-OHdG) (Cedarlane Labs, Burlington, ON) were all measured using enzyme-linked immunoassay (ELISA) according to manufacturer instructions.
2.2.3 **Wire Myography**

Segments of aortic rings (2 mm long) were mounted on a wire myograph for measuring isometric tension (DMT 620M, Danish Myotechnology, Aarhus, Denmark). Each myograph chamber contained PSS (in mM: NaCl (119), KCl (4.7), KH$_2$PO$_4$ (1.18), MgSO$_4$ (1.17), NaHCO$_3$ (24.9), EDTA (0.023), CaCl$_2$ (1.6) and dextrose (11.1)) kept at 37°C and pH 7.4 with continuous administration of 95% O$_2$ and 5% CO$_2$ gases. High KCl solution was prepared by equimolar substitution of NaCl in PSS. Aortic rings were stretched to their optimal tension (5.5 mN) then left to equilibrate for 20 mins before being challenged with 80 mM KCl and then returned to PSS again.

For endothelium-dependent vasodilation, aortic rings were preconstricted with a submaximal concentration of phenylephrine (Ph, 1 μM, Sigma) followed by cumulative additions of half-log concentrations ($10^{-10}$–$10^{-5}$ M) of acetylcholine (ACh, Sigma). For determining the role of basal nitric oxide production, two consecutive Ph concentration response curves were made, first in the absence and then after incubation with the endothelial nitric oxide synthase (eNOS) inhibitor $N_\omega$-nitro-$l$-arginine methyl ester hydrochloride (L-NAME, $10^{-4}$ M, Sigma). L-NAME inhibits eNOS to reduce intrinsic (basal) nitric oxide production, so causing a greater increase in Ph-induced vasoconstriction in proportion to the extent of basal nitric oxide produced. Basal nitric oxide production is estimated by the difference between the two PE curves and measured by the area under the curve (AUC) as we described elsewhere (122, 406).
2.2.4 Staining for endothelial nitric oxide synthase (eNOS) uncoupling

To investigate the role of eNOS as a source of superoxide anion, one of two aortic rings from the same animal was incubated in L-NAME (500 μM) for 30 mins at 37°C. Both rings were then embedded and cryosectioned (10 μm). Sections were then incubated with the fluorescent superoxide-sensitive dye dihydroethidium (DHE (1 μM), Molecular Probes) for 30 mins at 37°C in a humidity chamber. The reaction was stopped by placing the slides at 2-8°C for 20 mins. Slides were then cover slipped and fluorescence was detected (absorbance: 518 nm, emission: 605 nm) using an Olympus BX61 microscope with a RetigaEXi camera (QImaging, Surrey, Canada) and images were analyzed by the corrected total cell fluorescence (CTCF) method using ImageJ software (NIH, Bethesda, MD).

2.2.5 Western blotting

Fresh aortic tissues were homogenized in RIPA lysis buffer using a BeadBug homogenizer (Benchmark Scientific, Edison, NJ). Tissue homogenates were then transferred to sterilized filters with 0.8μm pores (Sartorius stedim biotech, Germany) and centrifuged for 10 minutes at 14,000xg to remove cell debris. Pierce bicichonic acid (BCA) assay was used to determine protein concentrations using manufacturer instructions (Thermo Fisher Scientific, Waltham, MA). For western blotting, 30μg of samples protein were loaded on polyacrylamide gels at 200 volts for 1 hour. The gels were then removed and transferred to nitrocellulose membranes overnight at 4°C using Mini-PROTEAN Tetra cell (Bio-Rad Laboratories, Hercules, CA). Membranes were then incubated for 1 hour with 5% non-fat milk (New England Biolabs, Ipswich, MA) for blocking, and then incubated with primary antibodies in tris-buffred saline (TBS 1L: 50 mM Tris-Cl, pH 7.6; 150 mM NaCl) with 0.05%
Tween-20 (Bio-Rad, Hercules, CA) (TBST) overnight at 4°C. The antibodies used were anti-DDAH2 (dimethylarginine dimethylaminohydrolase 2) rabbit monoclonal IgG at a dilution of 1:500 (Abcam, Cat# ab184166), anti-4-HNE modified proteins (4-hydroxynonenal) rabbit polyclonal IgG (Abcam Cat# ab46545, RRID: AB_722490) at a dilution of 1:500, and anti-ALDH2 (aldehyde dehydrogenase 2) mouse IgG (Santa Cruz Biotechnology Cat# sc-100496, RRID: AB_2242451) at a dilution of 1:100. Following overnight incubation, membranes were washed for 15 minutes three times with TBS, and the secondary goat anti-rabbit horseradish peroxidase-tagged antibody was added at a dilution of 1:2000 for DDAH2 and 4-HNE modified proteins (Abcam Cat# ab7090, RRID: AB_955417). The secondary mouse monoclonal IgGκ-binding protein was used for ALDH2 at a dilution of 1:1000 (Santa Cruz Biotechnology CAT# sc-516105, RRID: AB_2687626). After 1 hour of incubation at room temperature, detection was performed using an enhanced chemiluminescence kit Clarity Max (Bio-Rad, Hercules, CA).

### 2.2.6 Real time PCR

RNA was extracted using Qiazol lysis reagent (Qiagen, Hilden, Germany) and then purified using Isolated RNeasy mini kit (Qiagen, Hilden, Germany), according to manufacturer instructions. RNA quality and quantity were determined using Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA). Total RNA with ribosomal integrity number (RIN) > 7 were used for real-time PCR quantification by custom RT² Profiler™ PCR array (Qiagen, Hilden, Germany). Real-time PCR quantification was performed with an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) using
a custom made RT² Profiler PCR array consisting of SYBR® Green-optimized primer assays for 14 genes, including glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. cDNAs were synthesized from 0.5μg of total RNA using a commercial RT² First Strand Kit (Qiagen, Hilden, Germany) according to manufacture instructions. The synthesized cDNAs were then mixed with RT² q-PCR ROX master-mix containing SYBR green (Qiagen, Hilden, Germany). The mixture was then added to the custom RT² Profiler PCR array and qPCR was performed according to manufacturer instructions (Cycling conditions: 1 cycle 10 minutes 95°C to activate HotStart DNA Taq Polymerase followed by 40 cycles of 15 seconds 95°C and 1 minute 60°C). Data was analyzed using the integrated web-based automated software for RT² profiler PCR Array Data analysis (RT2 profiler PCR Array Data analysis version 3.5, GeneGlobe Data Analysis, SABiosciences). Gene expression fold changes were calculated using the ΔΔC_T method and the housekeeping gene control was used for normalization of the results. Fold-regulation values greater than 2 are indicated in red, fold-regulation values less than -2 are indicated in blue.

2.2.7 Statistical analysis

Values are expressed as means ± SD (n = 4-10). Vascular function data were recorded and analyzed by Powerlab 4/25 and Labchart 7 reader (AD instruments, Australia). Relaxations are expressed as percentage changes in tension from the pre-contraction to Ph; contractions are expressed as percentage of the reference response to 80 mM KCL. Gene expression data were assayed in triplicates to ensure the reliability of single values and statistical test was performed on ΔΔC_T values between groups, and data was expressed as fold
regulation represents fold-change in a biologically meaningful way. Two-way ANOVA with multiple comparisons followed by Bonferroni post hoc test was used to assess differences in the 4 groups; unpaired Student’s tests were used for real-time PCR and within-group analysis of tissues before and after L-NAME in the DHE staining experiments using Prism version 6.0 (GraphPad software, California, USA) $P$ value $<0.05$ was considered significant.
2.3 Results

2.3.1 Basic animal characteristics at the end of the experiment

As shown in Table 2-1, there were no significant changes in body weights, epididymal fat weight and fasting blood glucose due to CIH or dietary ALA. However, plasma insulin levels were lower in IHLA group when compared to IHRD group ($P<0.05$), likely due to the ability of ALA to improve insulin sensitivity (407).
Table 2-1: Characteristics of mice exposed to chronic intermittent hypoxia and treated with alpha lipoic acid compared to controls.

Values are displayed as mean ± SD and represent n = 5-6 mice. Statistical analysis was by two-way repeated measures ANOVA followed by Bonferroni post-hoc test. *P < 0.05 versus IARD, #P < 0.05 versus IALA, and $P < 0.05 versus IHRD. IARD: intermittent air regular diet, IALA: intermittent air with alpha lipoic acid, IHRD: intermittent hypoxia with regular diet, IHLA: intermittent hypoxia with alpha lipoic acid.

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Epidedimal fat (g)</th>
<th>Fasting blood glucose (mmol/L)</th>
<th>Plasma insulin (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IARD</td>
<td>36.7 ± 1.7</td>
<td>1.8 ± 0.3</td>
<td>7.9 ± 0.5</td>
<td>0.35 ± 0.05</td>
</tr>
<tr>
<td>IALA</td>
<td>33.3 ± 3.1</td>
<td>1.4 ± 0.6</td>
<td>7.5 ± 1.2</td>
<td>0.36 ± 0.04</td>
</tr>
<tr>
<td>IHRD</td>
<td>36.5 ± 4.1</td>
<td>1.9 ± 0.6</td>
<td>6.7 ± 0.9</td>
<td>0.44 ± 0.06</td>
</tr>
<tr>
<td>IHLA</td>
<td>33.7 ± 1.5</td>
<td>1.4 ± 0.2</td>
<td>6.4 ± 0.2</td>
<td>0.32 ± 0.07$</td>
</tr>
</tbody>
</table>
2.3.2 ALA improved endothelial dysfunction in mice exposed to CIH

Endothelium dependent relaxation was reduced in mice subjected to CIH compared to control \( (E_{\text{max}}: 55.2 \pm 3.8\% \) vs. \( 94.1 \pm 4.3\% \) of induced tone, \( P<0.0001 \)). Mice subjected to CIH and fed dietary ALA showed increased relaxation when compared to CIH alone \( (E_{\text{max}}: 80.1 \pm 6.2\%, P<0.0001) \) (Figures 2-1A, 2-1B). There was a significant interaction between ALA treatment and CIH exposure \( (P<0.0001, \text{Figure } 2-1B) \). However, dietary ALA decreased relaxation in control mice subjected to intermittent air alone, probably due to ALA acting as an anti-oxidant or pro-oxidant depending on the oxidant levels and physiological status (408). There were no significant changes in the EC\(_{50}\) for acetylcholine between all groups \( (P=\text{NS}) \).
Figure 2-1: Endothelium-dependent relaxation in mice exposed to CIH and treated with ALA

Cumulative concentration response curves to ACh in aortic rings preconstructed with Ph (A) and maximum relaxation response to ACh (B). Values are displayed as mean ± SD and represent n = 5-6 mice. Statistical analysis was by two-way repeated measures ANOVA followed by Bonferroni post-hoc test. *P < 0.05 versus IARD, #P < 0.05 versus IALA, and $P < 0.05 versus IHRD. ACh: acetylcholine, Ph: phenylephrine
2.3.3 ALA restored basal nitric oxide production in mice exposed to CIH

Basal production of nitric oxide maintains a vasodilatory tone in blood vessels at rest. Loss of that tone can lead to increase resting vasoconstriction and endothelial dysfunction. In the control and dietary ALA groups, the maximal contraction to phenylephrine was increased after incubation with L-NAME (% increase in $E_{max}$: 179.8 ± 14.1 and 168.9 ± 9.1, respectively) (Figures 2-2A, 2-2B). The modest increases in the maximum response in mice subjected to CIH after incubation with L-NAME were restored by dietary ALA (% increase in $E_{max}$: 123.1 ± 15.7 vs. 179.3 ± 8.1, $P<0.01$) (Figures 2-2C, 2-2D). Basal nitric oxide production was attenuated in mice subjected to CIH but was restored by ALA treatment (Figure 2-2E).
Figure 2-2: Basal NO production in mice exposed to CIH and treated with ALA

Cumulative concentration response curves to Ph before and after incubation aortic rings with L-NAME (A-D). AUC calculated for the contraction response to Ph after adding L-NAME (E). Values are displayed as mean ± SD and represent n = 5-6 mice. Statistical analysis was by two-way repeated measures ANOVA followed by Bonferroni post-hoc test. *P < 0.05 versus IARD, #P < 0.05 versus IALA, and $P < 0.05 versus IHRD. ACh: acetylcholine, AUC: area under the curve, L-NAME: Nω-nitro-L-arginine methyl ester, Ph: phenylephrine
2.3.4 Systemic oxidative stress and inflammation lowered in mice subjected to CIH and treated with ALA

Levels of urinary 8-OHdG, an oxidative stress marker for DNA damage, were higher in mice subjected to CIH when compared to control (1974.7 ± 627.1 vs. 578.7 ± 315.1 pg/ml, \( P<0.0001 \)) (Figure 2-3A). Dietary ALA decreased levels significantly in mice subjected to CIH (582.7 ± 201.6 pg/ml, \( P<0.0001 \)). Moreover, plasma levels of the inflammatory marker TNF-\( \alpha \) were higher in mice subjected to CIH (24.1 ± 4.9 vs. 5.4 ± 0.1 pg/ml, \( P<0.0001 \)) and lowered after ALA treatment (4.0 ± 1.6 pg/ml, \( P<0.0001 \)) (Figure 2-3B).
Figure 2-3: Plasma inflammatory and urinary oxidative stress markers

Plasma levels of TNF-α (A) and urinary levels of 8-OHdG (B). Values are displayed as mean ± SD and represent n = 5-10 mice. Statistical analysis was by two-way repeated measures ANOVA followed by Bonferroni post-hoc test. *P < 0.05 versus IARD, #P < 0.05 versus IALA, and $P < 0.05 versus IHRD. 8-OHdG: 8-hydroxy-2’-deoxyguanosine. TNF-α: tumor necrosis factor-α
2.3.5 ALA preserves eNOS coupling in mice subjected to CIH

Oxidative stress transforms eNOS from a coupled (nitric oxide producing) to an uncoupled (superoxide producing) state. Under normal physiological conditions, basal production of superoxide is scavenged by nitric oxide. As seen in the endothelial layer of control group (Figure 2-4A), increased fluorescence after incubation with the eNOS blocker (L-NAME) indicating prevention of superoxide scavenging by inhibiting nitric oxide production (CTCF: 100573 ± 22494 before L-NAME vs. 220384 ± 56462, \( P<0.0001 \)). However, decreased fluorescence after incubation with L-NAME in the mice subjected to CIH indicates eNOS uncoupling marked by reduced superoxide production (CTCF: 258053 ± 38225 vs. 146766 ± 30931, \( P<0.0001 \)). Treatment with ALA maintained eNOS in a coupled state since fluorescence was higher after incubation with L-NAME (CTCF: 122597 ± 28369 vs. 212614 ± 40729, \( P<0.0001 \)) (Figure 2-4B).
Figure 2-4: Uncoupled eNOS in the endothelium

Representative images of DHE staining of endothelial monolayer before and after incubation with L-NAME (20x)(A). Quantification of fluorescence levels using CTCF (B). Values are displayed as mean ± SD and represent n = 10 mice. Statistical analysis was by Student’s t-test within groups for comparison between groups before and after L-NAME. Two-way repeated measures ANOVA followed by Bonferroni post-hoc test was used for comparison between groups after L-NAME. *P < 0.05 before versus after L-NAME, #P < 0.05 versus IARD, $P < 0.05 versus IALA, and $P < 0.05 versus IHRD. CTCF: corrected total cell fluorescence, DHE: dihydroethidium, L-NAME: iNOS-nitro-L-arginine methyl ester
2.3.6 ALA decreased levels of ADMA in mice exposed to CIH

Plasma levels of ADMA, an endogenous competitive inhibitor of L-arginine (409), significantly increased in mice exposed to CIH when compared to control (0.76 ± 0.12μm vs. 0.31 ± 0.07μm, \( P<0.0001 \)). Treatment with ALA decreased ADMA levels (0.39 ± 0.13μm, \( P<0.001 \)) (Figure 2-5A). Changes in plasma ADMA levels occurred with no changes in the aortic expression of DDAH-2, the enzyme responsible for metabolizing ADMA (Figure 2-5B).
Figure 2-5: Levels of ADMA, an endogenous inhibitor of eNOS levels, and expression of DDAH2, its metabolizing enzyme

Plasma levels of ADMA (A), aortic expression of DDAH2 (B). Values are displayed as mean ± SD and represent n = 4-10 mice. Statistical analysis was by two-way repeated measures ANOVA followed by Bonferroni post-hoc test. *P < 0.05 versus IARD, #P < 0.05 versus IALA, and $P < 0.05 versus IHRD. ADMA: asymmetric dimethylarginine, DDAH2: dimethylarginine dimethylaminohydrolase 2
2.3.7 ALA increased levels of ALDH2 and decreased levels of 4-HNE

ALDH2 is a mitochondrial enzyme that metabolizes acetaldehyde and detoxifies reactive aldehydes, such as 4-HNE, that are generated from lipid peroxidation caused by oxidative stress (410). Aortic expression of ALDH2 was not affected in mice exposed to CIH when compared to control (Figure 2-6A). However, expression of 4-HNE modified proteins was higher in CIH group. Treatment with ALA increased ALDH2 expression and decreased 4-HNE modified protein expression in mice exposed to CIH (Figure 2-6B).
Figure 2-6: ALDH2 and 4-HNE protein adducts levels

Aortic protein expression of ALDH2 (A) and 4-HNE protein adducts (B). Values are displayed as mean ± SD and represent n = 5-6 mice. Statistical analysis was by two-way repeated measures ANOVA followed by Bonferroni post-hoc test. *P < 0.05 versus IARD, #P < 0.05 versus IALA, and $P < 0.05 versus IHRD. 4-HNE: 4-hydroxynonenal, ALDH2: aldehyde dehydrogenase 2.
2.3.8 ALA up-regulated antioxidant enzymes and blunted inflammatory cytokine gene expression in mice subjected to CIH

Aortic mRNA expression of inflammatory cytokines was increased in mice exposed to CIH when compared to control, while the expression of antioxidant genes was not affected (data not shown). Mice exposed to CIH and treated with ALA had increased gene expression of antioxidant enzymes (11- to 66-fold) and reduced expression of inflammatory cytokines (8- to 34-fold) when compared to mice exposed to CIH alone (Table 2-2).
Table 2-2: Fold changes of gene expression in aortic tissue of mice subjected to chronic intermittent hypoxia compared to mice treated with alpha lipoic acid

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<thead>
<tr>
<th>Inflammatory cytokines</th>
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<tr>
<td></td>
<td><em>Il6</em></td>
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<td><em>Mcp1</em></td>
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<td><em>Vcam1</em></td>
<td>Vascular cell adhesion molecule 1</td>
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<table>
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<td><em>Sod1</em></td>
<td>Superoxide dismutase 1</td>
<td>11*</td>
</tr>
<tr>
<td></td>
<td><em>Gpx3</em></td>
<td>Glutathione peroxidase 3</td>
<td>29*</td>
</tr>
<tr>
<td></td>
<td><em>Hmox1</em></td>
<td>Heme oxygenase 1</td>
<td>38*</td>
</tr>
<tr>
<td></td>
<td><em>Nqo1</em></td>
<td>NAD(P)H dehydrogenase</td>
<td>66*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ROS producing enzymes</th>
<th>Gene Symbol</th>
<th>Name of gene</th>
<th>Fold-regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Nox1</em></td>
<td>NADPH oxidase 1</td>
<td>-2</td>
</tr>
<tr>
<td></td>
<td><em>Nox4</em></td>
<td>NADPH oxidase 4</td>
<td>-2</td>
</tr>
</tbody>
</table>

Student’s unpaired t test was performed on ΔΔC\textsubscript{T} values between groups; Values are displayed as mean ± SD and represent n = 5 mice. *P < 0.05 versus IHRD. Fold regulation represents fold-change in a biologically meaningful way. Fold-regulation values greater than 2 are indicated in red, fold-regulation values less than -2 are indicated in blue, IHRD: intermittent hypoxia regular diet, IHLA: intermittent hypoxia lipoic acid.
2.4 Discussion

We demonstrated that dietary ALA treatment attenuated endothelial dysfunction in mice subjected to CIH as indicated by improvements in ACh-induced vasodilation and basal nitric oxide production. We also show that ALA 1) decreased oxidative DNA damage and inflammatory marker levels in urine and plasma 2) prevented mRNA expression of inflammatory markers in aortic tissue 3) increased mRNA expression of antioxidant enzymes in aortic tissue and 4) maintained eNOS in a coupled state. Findings of this study are summarized in Figure 2-7.
Figure 2-7: Summary of antioxidant and anti-inflammatory effects of ALA in mice exposed to CIH.

OSA is associated with oxidative stress, inflammation and endothelial dysfunction (5). We and others have reported endothelial dysfunction caused by CIH in mouse aorta, (405) and other vascular beds such as rat cerebral and skeletal muscle arteries (411). The extent of endothelial dysfunction depends on the intensity and duration of CIH (412). Although CPAP can reverse endothelial dysfunction in OSA subjects, (413) patient adherence limits its use (414). Treatment of OSA subjects with intravenous injection of 0.5g vitamin C acutely improved endothelial function (measured by flow-mediated dilation), suggesting a role for antioxidant treatment of OSA-related CVD (415). This study however, had a small sample size (n=10) and treatment was only one injection of vitamin C without controlling for body weight. Treating rodents with xanthine oxidase (162) reduced endothelial dysfunction caused by CIH but had no effects on oxidative stress markers. We used ALA, a readily available, relatively safe agent to improve endothelial function; this compound has both antioxidant and anti-inflammatory effects (166). Our data show that ALA ameliorates endothelial dysfunction in mice subjected to CIH. Clinical and animal studies confirm that ALA improves endothelial function in several other diseases (400, 402, 416). Conversely, ALA treatment in mice subjected to intermittent air resulted in reduced endothelial function when compared to intermittent air with regular diet. Some studies reported pro-oxidant effects of ALA only in animal studies, likely due to higher plasma concentrations than those after oral or intravenous infusion of ALA in humans (417, 418). In our study, we did not observe any increase in oxidative stress or inflammation in ALA treated control. We suggest that the inhibition of endothelium-dependent vasodilation is independent on ALA’s pro-oxidant effect. The controversial outcomes of ALA treatment arise from the dose, route of
administration, enantiomer used, disease, and duration of treatment (419). It is essential to evaluate all these factors in clinical use to avoid the adverse effects of ALA.

Oxidative stress and inflammation are important mechanisms of endothelial dysfunction in OSA and CVD (420). Oxidative stress is characterized by an imbalance between anti-oxidant system and pro-oxidant systems leading to accumulation of reactive oxygen species (ROS). CIH in OSA leads to increased ROS production and impairment of antioxidant capacity (421). Increased ROS production interacts with nitric oxide to decrease its bioavailability and produces a potent reactive nitrogen free radical (peroxynitrite) that oxidizes lipids, proteins and DNA (395). A recent study of OSA subjects concluded that 2 months of CPAP treatment did not reduce oxidative stress markers despite CPAP adherence (422). Our study demonstrated that urinary levels of 8-OHdG are higher in mice subjected to CIH, similar to findings in OSA subjects (81). Treatment with dietary ALA not only lowered systemic oxidative stress, but also increased aortic mRNA levels of antioxidant enzymes in mice exposed to CIH. Indeed, ALA can directly act as an antioxidant, regenerate and maintaining endogenous antioxidants and activating nuclear factor (erythroid-derived 2)-like 2 (Nrf2, a key transcription factor that mediates the expression of antioxidant and detoxification genes regulated by the antioxidant response element (ARE)) (423).

ADMA is a naturally occurring L-arginine analog derived from proteolysis of methylated protein, and the enzyme DDAH prevents its accumulation. However, in pathological conditions such as chronic kidney failure (424), ADMA levels are elevated and can compete with L-arginine for the binding site in the active center of NOS and thus inhibit the production of nitric oxide (409). ADMA is an independent risk factor for coronary heart
disease according to the multicenter Coronary Artery Risk Determination investigating the Influence of ADMA Concentration (CAR-DIAC) study (425). Others have reported that ADMA levels are elevated in subjects with OSA and mice exposed to CIH (405, 426). In our study, treatment of mice exposed to CIH with ALA decreased levels of ADMA without affecting DDAH-2 expression in the aorta. Oxidative stress can reduce the activity and can cause ADMA levels to increase (427). ALA as an antioxidant might have protected DDAH2 from oxidation, which could account for the decreased levels of ADMA in CIH group.

ALDH2 is the mitochondrial form of aldehyde dehydrogenase responsible for metabolism of toxic aldehydes and ROS-generated aldehyde adducts that can adduct with lipids, proteins and DNA, leading to their inactivation (410, 428). ALDH2 activity involves cysteine thiol groups that are susceptible to oxidative stress and so render the enzyme inactive (429). ALA (and its reduced form) can restore the activity of oxidized ALDH2 (430, 431). Our results show that ALDH2 expression was not affected by CIH but treatment of mice exposed to CIH with ALA significantly increased ALDH2 expression in aortic tissue. Furthermore, CIH increased 4-HNE modified proteins expression in aorta but treatment with ALA decreased the expression these proteins significantly, indicating that ALA may have enhanced 4-HNE detoxification through increasing ALDH2 activity.

Inflammation is prominent in OSA and is responsible for initiation of atherosclerosis in CVD (432). Oxidative stress activates transcription factor NF-κB, causing it to translocate to the nucleus where it initiates the transcription of various inflammatory cytokines such as interlukin-6 (IL-6) and TNF-α and endothelial adhesion molecules (433). Recent clinical trials reported that CPAP did not improve levels of C-reactive protein (CRP), IL-6 and TNF-α
in OSA subjects. (240, 434) We show that plasma levels of TNF-α and mRNA expression of inflammatory cytokines such as TNF-α, IL-6 and monocyte attractant protein 1 (MCP-1) are increased by CIH, and that dietary ALA reversed systemic and aortic inflammation in mice subjected to CIH. This effect is likely due to the ability of ALA to prevent the translocation of NF-κB (435) independent of its antioxidant mechanisms (176).

OSA uncouples eNOS in the vasculature, causing production of superoxide anion instead of nitric oxide. Oxidative stress leads to the oxidation of tetrahydrobiopterin (BH₄), an essential cofactor essential for NO production, leads to eNOS uncoupling. Supplementation with BH₄ reverses endothelial dysfunction in OSA subjects (436). We evaluated eNOS uncoupling in aortic sections by measuring the fluorescence of the superoxide-sensitive dye (DHE) before and after incubation with L-NAME (eNOS inhibitor). Increased fluorescence in control mice after L-NAME indicates decreased nitric oxide availability for interaction with superoxide anion. On the other hand, decreased fluorescence after L-NAME incubation indicates blockage of uncoupled eNOS due to lower amounts of superoxide anion, as seen in mice subjected to CIH. However, ALA treatment in mice subjected to CIH increased fluorescence after L-NAME incubation, indicating that eNOS was preserved its coupled state.

In summary, our findings suggest ALA as a potential therapeutic supplement for improving cardiovascular outcomes in OSA subjects.
Chapter 3: Gestational intermittent hypoxia impairs uterine artery function in pregnant mice

3.1 Introduction

Obstructive sleep apnea is a chronic condition characterized by repetitive episodes of complete or partial collapse of the upper airway leading to chronic intermittent hypoxia (IH), intrathoracic pressure swings and sleep fragmentation (2). It is well recognized that OSA is a risk factor for many non-communicable diseases such as hypertension, diabetes and cardiovascular disease (CVD) (437). Increases in inflammation (438), oxidative stress (395), and sympathetic activation (439) are proposed to link OSA to CVD, as these mechanisms lead to vascular endothelial dysfunction and progression of atherosclerosis (440).

Pregnancy can induce SDB conditions such as snoring and OSA that worsens with the progression of pregnancy. Snoring (3 or more nights per week) increases from 7% to 11% in the first trimester to 16% to 25% in the third trimester (182). Prevalence of OSA in pregnant women (as measured by the apnea-hypopnea index (AHI)) increases from early in pregnancy (3.6%) to mid-pregnancy (8.3%) (441). Indeed, OSA leads to fetoplacental hypoxia (442) and increases oxidative stress and inflammatory biomarkers in pregnant women (443). OSA in pregnancy is also associated with gestational hypertension and preeclampsia (PE) (212), gestational diabetes (444) and adverse foetal outcomes (222). However, the effects of pre-existing OSA on fetoplacental outcomes remain unknown due to lack of clinical and experimental studies.

The uteroplacental circulation undergoes continuous remodelling throughout pregnancy including: i) increased diameter of uterine arteries, ii) vasculo/angiogenesis, and
iii) remodelling of spiral arteries (445). These changes ensure adequate oxygen and nutrient supply to the foetus. Defects in vascular adaptations can result in severe maternal and foetal complications such as PE and intrauterine growth restriction (IUGR) (446). In this study, we hypothesized that gestational intermittent hypoxia (GIH) causes endothelial dysfunction in uterine arteries of pregnant mice dams. Our aims were to assess the impact of GIH on spiral artery remodelling, levels of circulating angiogenic and anti-angiogenic factors, and placental oxidative stress at day 14.5 of pregnancy.

3.2 Materials and Methods

3.2.1 GIH in pregnant dams

Experiments were approved by the Animal Care Center at the University of British Columbia, Canada (Certificate number: A17-0140). Male and female C57BL/6J mice (8 weeks old) were purchased from Charles River (Wilmington, MA) and kept in a controlled environment with 12h light/dark cycles with ad libitum access to normal chow diet and water. The copulating protocol involved introducing two female mice to one male mouse in each cage. After visualization of a copulation plug (day 0.5 of pregnancy), females were separated into two groups: [1] pregnant dams subjected to intermittent hypoxia (IH) and [2] pregnant dams subjected to room air (IA). Briefly, pregnant dams were placed in specialized cages with ports evenly spaced at the bottom of the cages to regulate levels of gas/air inside the cages. The IH profile consists of alternate cycles of fraction of oxygen inspired ($F_{iO_2}$) of 21-12% using nitrogen ($N_2$) and compressed air. Pregnant dams were exposed to IH for 60 cycles/h during the light cycle (12h) with oxyhemoglobin desaturation levels of ~75% per
cycle. For the groups exposed to IA, pregnant dams were exposed to room air (FiO₂ 21%) only (122). At day 14.5 of pregnancy (resembles the beginning of the third trimester in human pregnancy), IH was halted and the mice were fasted for 4 hours and injected with hypoxyprobe-1 (Hypoxyprobe, Inc. Burlington, MA) to assess placental hypoxia 1 hour before euthanasia.

3.2.2 Plasma and tissue collection

Pregnant dams were euthanized using an overdose of the inhalant anesthetic isoflurane (5% at 1-2 L O₂/min) followed by carbon dioxide. Uterine arteries were collected and dissected in ice-cold oxygenated physiologic salt solution (PSS). Blood samples were drawn from the inferior vena cava using heparinized syringes and transferred to Eppendorf tubes. The blood was centrifuged (10 min at 4 °C, 1000g) for plasma separation; plasma samples were stored at −80 °C in aliquots after snap freezing in liquid nitrogen. Placentas were collected and fixed in 4% paraformaldehyde (PFA) for 24 hours followed by 70% ethanol for fixation.

3.2.3 Vascular reactivity

Cleaned uterine arteries were cut into equal 2 mm rings and mounted on a wire myograph for measuring isometric tension (DMT 620M, Danish Myotechnology, Aarhus, Denmark) using stainless steel wires (25μm). Each myograph chamber contained PSS (in mM: NaCl (119), KCl (4.7), KH₂PO₄ (1.18), MgSO₄ (1.17), NaHCO₃ (24.9), EDTA (0.023), CaCl₂ (1.6) and dextrose (11.1)) kept at 37°C and pH 7.4 with constant administration of 95%
O$_2$ and 5% CO$_2$. Blood vessels were stretched to their optimal tension during the normalization procedure. Each arterial ring was stretched in a stepwise manner. Wall tension and internal circumference after each stretch was calculated and plotted to provide a resting wall tension-internal circumference curve for that artery, using the DMT Normalization Module. Arterial rings were normalized to 0.9·L$_{100}$, where L$_{100}$ is the internal circumference of the vessels at a transmural pressure of 100 mmHg. Optimal diameters (OD) were calculated as OD = 0.9·L$_{100}$/π (447). After obtaining the OD, vessel rings were allowed to equilibrate for 30 mins before being challenged twice with 80 mM KCl and then rested in normal PSS again. After resting, constrictions to cumulative additions of phenylephrine (Ph, $10^{-9}$–$10^{-5}$ M) were recorded. The responses to Ph were expressed as the percentage of maximal contraction induced by KCl. For endothelium-dependent vasodilation, uterine artery segments were preconstricted with a submaximal dose of Ph (1 μM) followed by cumulative additions of half-log concentrations of acetylcholine (ACh, $10^{-9}$–$10^{-5}$ M)). To assess the contribution of nitric oxide (NO) to vasodilation, arteries were incubated with the endothelial nitric oxide synthase inhibitor (eNOS) N$_ω$-nitro-l-arginine methyl ester hydrochloride (L-NAME, $10^{-4}$ M) for 45 minutes before preconstruction with Ph and addition of ACh. Cumulative concentrations of sodium nitroprusside (SNP, $10^{-9}$–$10^{-5}$ M) after preconstriction with Ph (1 μM) were used to assess endothelium-independent relaxation.

### 3.2.4 Biochemical measures

Plasma 8-isoprostane (Cayman Chemical, Ann Arbor, MI) and tumor necrosis factor-α (TNF-α, R&D systems, Minneapolis, MN) were measured using enzyme-linked
immunosorbent assay (ELISA). Plasma angiogenic and anti-angiogenic factors such as vascular endothelial growth factor (VEGF), placenta growth factor-2 (PIGF-2), soluble fms-like tyrosine kinase-1 (sFlt-1 or sVEGFR-1) and soluble endoglin (sENG) were also measured using ELISA (R&D systems, Minneapolis, MN). All assays were performed according to manufacturer’s instructions.

3.2.5 Placental morphometry, immunohistochemistry and in situ cell death

Placental tissues were embedded in paraffin blocks after fixation and cut into 5 µm sections. Sections were then deparaffinised and rehydrated using xylene and downgraded concentrations of alcohol, after which slides were immersed in 10 mM sodium citrate (pH 6) for 20 minutes in a steam bath (95 °C) to allow for antigen retrieval. Immunostaining was performed using avidin biotin complex (ABC) IHC kit (Abcam, Cat# ab64261). Placental sections were incubated with the following antibodies: rabbit polyclonal anti-alpha smooth muscle actin (Abcam, Cat# ab5694, RRID: AB_2223021) and rabbit monoclonal anti-cytokeratin 7 (Abcam, Cat# ab181598) for detection of spiral artery remodelling, and rabbit polyclonal anti-4 hydroxynonenal (4-HNE, Abcam Cat# ab46545, RRID: AB_722490) for assessment of placental oxidative stress. Sections were then washed and incubated with biotinylated goat anti-rabbit IgG included in the kit (H&L) for 1 hour. Streptavidin peroxidase was then applied for 10 minutes followed by 20ul DAB chromogen for 10 minutes with washing between steps. Finally, sections were counterstained with hematoxylin (Santa Cruz Biotechnology, CAS 517-28-2) and covered with mounting medium. Twenty images per placenta were taken for analysis using IHC profiler plugin within Image J software (National Institute of Health) and values expressed as percent of highly positive staining. Periodic acid-
Schiff (PAS) and Haematoxylin & Eosin (H&E) stains were performed on three sections/placenta with 100μm spacing to assess placental compartment sizes using Aperio ImageScope (Leica Biosystems, Germany). Labyrinth and junctional zone areas were calculated as percentages of total placental area. ImageScope was also used to measure lumen area of spiral arteries. Assessment of In situ cell death was detected using ApopTag® Peroxidase In Situ Apoptosis Detection Kit according to manufacturer instructions (EMD Millipore, Burlington, MA)(448). Images were examined under a light microscope and photographed using an Olympus BX61 light microscope or whole slide scanned using Leica ScanScope (Leica Biosystems, Germany).

### 3.2.6 Placental hypoxia

Hypoxprobe-1 kit contains pimonidazole hydrochloride and mouse monoclonal antibody for detection of pimonidazole in hypoxic tissue. Pimonidazole binds to thiol-containing proteins in hypoxic cells (449). Pregnant mice were injected intraperitoneally at day 14.5 of gestation with pimonidazole hydrochloride (60 mg/kg maternal body weight). One hour later, the mothers were euthanized, and placentas were fixed in 4% PFA for 24 h followed by 70% ethanol. Placentas were embedded in paraffin after the fixation, cut into 5-μm sections, and immunostained for pimonidazole following the manufacturer’s protocol (450).
3.2.7 Statistical analysis

Dose-response curves were fitted by nonlinear regression with simple algorithm. Relaxations are expressed as percentage changes in tension from the pre-contraction to Ph; contractions are expressed as percentage of the reference response to 80 mM KCL. Vascular function data were recorded and analyzed by Powerlab 4/25 and Labchart 7 reader (AD instruments, Australia). Unpaired Student’s $t$-test was to assess difference between groups using Prism version 6.0 (GraphPad software, California, USA). One-way ANOVA with multiple comparisons followed by Bonferroni post hoc test was only used to compare dose-response curves of ACh with and without L-NAME. $P$ value $<0.05$ was considered significant.
3.3 Results

3.3.1 Increased placental and low foetal weights in pregnant mice exposed to GIH

Maternal weights, foetal weights, and pup numbers were all significantly lower in IH pregnant dams (Table 3-1), but placental weights were significantly higher in IH dams (Table 3-1). However, food consumption between the two groups did not differ. Percentage of resorption sites was higher in IH dams while pup-to-placental ratio, as an indirect measure of placental insufficiency, was significantly lower (Table 3-1).
Table 3-1: Physiological characteristics of pregnant mice exposed to GIH and control at day 14.5 of gestation

<table>
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<tr>
<th>Characteristics</th>
<th>IA</th>
<th>IH</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mothers body weight (g)</td>
<td>17.6 ± 1.1</td>
<td>16.9 ± 1.1</td>
<td>0.26</td>
</tr>
<tr>
<td>Food consumption (g/day)</td>
<td>4.9 ± 1.1</td>
<td>4.7 ± 0.6</td>
<td>0.79</td>
</tr>
<tr>
<td>Pup weight (g)</td>
<td>0.23 ± 0.02</td>
<td>0.17 ± 0.01</td>
<td>0.001</td>
</tr>
<tr>
<td>Pup number</td>
<td>8.8 ± 0.7</td>
<td>6.8 ± 0.7</td>
<td>0.001</td>
</tr>
<tr>
<td>Placental weight (g)</td>
<td>0.09 ± 0.002</td>
<td>0.11 ± 0.004</td>
<td>0.002</td>
</tr>
<tr>
<td>Resorption (%)</td>
<td>3.1 ± 0.58</td>
<td>24.6 ± 9.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Pup/placenta weight ratio</td>
<td>2.60 ± 0.3</td>
<td>1.5 ± 0.1</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Data are represented as mean ± SD, n = 5-6. IH: intermittent hypoxia, IA: intermittent air
3.3.2 GIH impairs uterine artery function in pregnant mice

There was no difference in uterine artery diameters between the two groups (246 ± 15μm (IH) vs. 241± 15μm (IA), P=NS). Endothelium-dependent relaxation response to ACh was decreased significantly in uterine arteries of IH pregnant dams (Figure 3-1A) with a lower maximal response to ACh ($E_{max}$ 63.3 ± 3.7% (IH) vs. 93.9 ± 2.8% (IA), P<0.001) (Figure 1B). Incubation with L-NAME abolished relaxation responses to ACh in both groups (IA $E_{max}$ 13.2 ± 4.6%, IH $E_{max}$: 10.13 ± 2.8) (Figure 3-1A, 3-1B). Relaxation response was completely abolished when uterine arteries from both groups were incubated with L-NAME and indomethacin (5*10^-5 M, inhibits prostaglandin synthesis). This indicates that prostaglandins are responsible for the L-NAME-insensitive relaxation response (data not shown). The constrictor response to phenylephrine was significantly higher in IH dams ($E_{max}$ 167.6 ± 13.9%$K_{max}$ vs. 116 ± 11.05%$K_{max}$, P=0.0017) (Figure 3-1C). Endothelium-independent relaxation responses to SNP were similar in IH and IA exposed dams (P=NS) (Figure 3-1D).
Figure 3-1: Impaired endothelium-dependent relaxation in uterine arteries of pregnant mice exposed to GIH

Concentration-response curves to acetylcholine (ACh) with and without the addition of L-NAME (A) and maximal responses to ACh (B), concentration-response curves to Ph (C) and SNP (D) from IH and IA pregnant dams. Values are displayed as mean ± SD and represent n = 5-6 mice. Responses to ACh before and after L-NAME were compared using one-way ANOVA followed by Bonferroni post-hoc test. Responses to Ph and SNP were compared using Student’s unpaired t-test. *P<0.05 vs. IA, #P<0.05 vs. IH. ACh: acetylcholine, L-NAME: Nω-nitro-L-arginine methyl ester, Ph: phenylephrine
3.3.3 Increased systemic oxidative stress and inflammation in pregnant mice exposed to GIH

The oxidative stress marker 8-isoprostane was higher in plasma from IH pregnant dams (91.1 ± 9.6pg/ml vs. 52.4 ± 11.9pg/ml, $P=0.0005$) (Figure 3-2A). Similarly, the inflammatory cytokine TNF-α plasma levels were higher in IH (16.5 ± 2.9pg/ml vs. 5.9 ± 1.3pg/ml, $P<0.0001$) (Figure 3-2B).
Figure 3-2: Systemic oxidative stress and inflammation is increased in pregnant mice exposed to GIH

Plasma 8-Isoprostane (A) and TNF-α (B) in IH and IA pregnant dams. Values are displayed as mean ± SD and represent n = 5-6 mice. Groups were compared using Student's unpaired t-test. *P<0.05 vs. IA, TNF-α: tumor necrosis factor-α
3.3.4 Increased circulating anti-angiogenic factors in pregnant mice exposed to GIH

Plasma levels of sFlt-1 were significantly higher in IH dams (15.8 ± 2.6 ng/ml vs. 4.8 ± 1.1 ng/ml, \( P<0.0001 \)) (Figure 3-3A). Likewise, plasma levels of sEng (2122 ± 225 pg/ml vs. 1301 ± 109 pg/ml, \( P<0.0001 \)) (Figure 3-3B), PIGF-2 (111.2 ± 15.6 pg/ml vs. 160.2 ± 40.3 pg/ml, \( P=0.03 \)) (Figure 3-3C) and VEGF (146.5 ± 35.4 pg/ml vs. 477.8 ± 44.5 pg/ml, \( P<0.0001 \)) were also all higher in IH dams (Figure 3-3D).
Figure 3-3: Circulating angiogenic and anti-angiogenic factors in pregnant mice exposed to GIH.

Plasma sFlt-1 (A), sENG (B), PIGF-2 (C) and VEGF (D) in IA and IH pregnant dams. Values are displayed as mean ± SD and represent n = 5 mice. Groups were compared using Student’s unpaired t-test. *P<0.05 vs. IA.

sFlt-1: soluble fms-like tyrosine kinase-1, sENG: soluble endoglin, PIGF-2: placental growth factor-2, VEGF: vascular endothelial growth factor
3.3.5 **Increased placental labyrinth size of pregnant mice exposed to GIH**

PAS staining was used to distinguish placental compartments (Figure 3-4A). The ratios of labyrinth size to whole placenta size were larger in IH dams (44.6 ± 3.2% vs. 36.5 ± 2.4% *P*<0.0001) (Figure 3-4B). Junctional zone sizes were not significantly different between IH and IA (*P*=NS) (Figure 3-4C).
Figure 3-4: Placental labyrinth and junctional zone sizes in pregnant mice exposed to GIH

PAS staining of whole placenta (1.5x) (A), percentage of placental labyrinth size (B), percentage of placental junctional zone size (C) in IA and IH pregnant dams. Values are displayed as mean ± SD and represent n = 5-6 mice. Groups were compared using Student’s unpaired t test. (Three sections/placenta, 100 micron apart).

*P<0.05 vs. IA. PAS: Periodic acid-Schiff
3.3.6 GIH did not impair spiral artery remodelling in pregnant mice

Staining with α-SMC (for detection of smooth muscle cells) was absent in spiral arteries while expression of CK7 for trophoblasts was apparent (Figure 3-5A). Furthermore, the lumen area of spiral arteries in placental sections was not different between IH and IA (20886 ± 1626μm² vs. 17971 ± 6492 μm², \( P=NS \)) (Figure 3-5B).
Figure 3-5: Spiral artery remodeling in pregnant mice exposed to GIH

α-SMA and CK7 staining (20x) (A), lumen area of spiral arteries (B) in IA and IH pregnant dams. Values are displayed as mean ± SD and represent n = 5 mice. Groups were compared using Student’s unpaired t-test.

*P<0.05 vs. IA. α-SMA: α-smooth muscle actin, CK7: cytokeratin-7
3.3.7 Increased placental hypoxia, oxidative stress and cell death in pregnant mice exposed to GIH

Immunostaining of hypoxia in placenta, as shown by pimonidazole reaction (Figure 3-6A), was higher in IH dams (2.2 ± 1.1 vs. 8.9 ± 2.1, $P=0.0014$) (Figure 3-6B). Furthermore, oxidative stress, as indicated by immunostaining of 4-HNE adducts, was higher placenta of IH pregnant mice (1.6 ± 0.2 vs. 3.8 ± 0.4, $P<0.0001$) (Figure 3-6C). Moreover, TUNEL staining, as an indicator of cell death, was higher placentas of IH pregnant dams (0.56 ± 0.14 vs. 0.98 ± 0.1, $P=0.0012$) (Figure 3-6D).
Figure 3-6: Placental hypoxia, oxidative stress and cell death in pregnant mice exposed to GIH.

Pimonidazole (5x), 4-HNE (5x) and TUNEL staining in placental sections (20x) (A), percentage of highly positive staining of pimonidazole (B), 4-HNE (C) and percentage of highly positive nucleus staining of TUNEL (D) in IA and IH pregnant dams. Values are displayed as mean ± SD and represent n = 5 mice. Groups were compared using Student’s unpaired t-test. *P<0.05 vs. IA. 4-HNE: 4-hydroxynonenal
3.4 Discussion

Our study is the first to assess the effects of GIH on uteroplacental circulation and placental outcomes in pregnant mice, where we report: 1) low foetal weight and increased placental weight and number of resorption sites, 2) enhanced uterine artery tone caused by impaired endothelium-dependent relaxation and increased vasoconstriction, 3) increased systemic oxidative stress and inflammation, 4) increased circulating angiogenic and anti-angiogenic factors, 5) increased placental hypoxia, oxidative stress and cell death (Figure 3-7).
Figure 3-7: Impact of GIH on uterine arteries and placenta of pregnant mice exposed to GIH

$FiO_2$: fraction of oxygen expired, PIGF-2: placental growth factor 2, sFlt-1: soluble vascular endothelial growth factor receptor 1, sENG: soluble endoglin, VEGF: vascular endothelial growth factor.
Prospective and retrospective studies report that OSA reduces foetal growth in late pregnancy (222, 451). Proper management with continuous positive airway pressure (CPAP) is able to prevent foetal weight loss. However, there are also reports that OSA does not affect foetal weight and even accelerates foetal growth during the third trimester (452). Conflicting results may stem from non-objective tools used for assessment and inadequate control of confounding factors (452). However, all of the studies mentioned have examined the effects of pregnancy-associated OSA with no reports on effects of pre-existing OSA on maternal/foetal outcomes. Our study shows that exposing pregnant mice to GIH reduced foetal weights at day 14.5 of pregnancy. Under normal conditions, placental weight increases as does birth weight, but in some situations a reduced placental weight to birth ratio results in adverse pregnancy outcomes (453). Thus, offspring of patients with preeclampsia have lower birth weights and lower placental weights (454). On the other hand, normobaric sustained hypoxia in pregnant mice (13% $FiO_2$) results in foetal growth restriction and increased placental weight at day 18.5 of pregnancy (455). We report increased placental weight to body weight ratios at day 14.5 in pregnant mice exposed to GIH, suggesting placental insufficiency with increased placental labyrinth area, possibly to allow for increased surface area for gas exchange (447). This effect seems to be independent of maternal nutrition since there was no change in food consumption between the two groups. Foetal oxygenation is a major regulator of foetal growth, likely due to the depressive effects of oxygen deprivation on ATP synthesis and/or endocrine factors thought to be important in foetal growth (300).
The cardiovascular system undergoes extensive adaptations during pregnancy to allow for the increased demand of blood flow to the fetoplacental unit required to maintain a normal pregnancy (300). Vascular resistance drops by 35%-40% preceding the development of the placenta through enhanced vasodilation and reduced vasoconstriction (456), facilitating for an increase blood volume by 140% during pregnancy (457). Perturbation in vascular adaptations during pregnancy leads to maternal and foetal complications (458). Uterine arteries branch from the internal iliac artery anastomose with the ovarian artery to form a circulatory loop. In mice, the diameter of uterine arteries increases by 55% in early gestation and continues to increase with the progression of pregnancy (459). Uterine artery dysfunction occurs in women (459) and animals with PE (460, 461) and also after exposure to sustained hypoxia (462).

Our study is the first to assess uterine artery function in a mouse model of OSA. We show that that endothelium-dependant relaxation in response to ACh was attenuated in pregnant mice exposed to GIH. Incubation with eNOS blocker (L-NAME) abolished responses to ACh in both groups indicating a major role for NO in regulating uterine arterial tone, and is supported by studies in eNOS knockout mice where there is a 30% reduction in uterine artery diameter and reduced placental blood flow (463). However, the relative importance of the NO pathway is likely to be different in smaller uterine vascular beds such as radial, arcuate and spiral arteries (464). We also demonstrated increased constriction of uterine arteries to the α1-agonist phenylephrine. Thus, the combined effects of impaired vasodilation and increased vasoconstriction due to GIH can decrease blood flow and adversely affect the fetoplacental unit.
The uterine artery branches into radial arteries that further divide into arcuate branches that extend into the internal endometrial layer, where it branches further into basal arteries that eventually form the spiral arteries that supply the endometrial layer. As the decidua develops, the spiral arteries undergo extensive remodelling, changing from arteries with high resistance and low capacity to those with low resistance and high capacity, so allowing increased exchange of nutrients and oxygen with the foetal circulation. The remodelling process involves natural killer cells, trophoblast invasion into the vascular wall and apoptosis of smooth muscle cells (445). This remodelling process reduces total vascular resistance in the mouse uterine bed by 47% (459). Insufficient remodelling is thought to be the main cause of complicated pregnancies in PE (465, 466). In our study, GIH did not alter spiral artery remodelling, as shown by the absence of α-SMA with clear trophoblast marker staining (CK7) in immunohistochemical studies. Furthermore, the luminal area of spiral arteries was unaltered by GIH. Maternal hypoxia can be adaptive or detrimental depending on magnitude, duration, and the gestational timing of hypoxic conditions (467). We speculate that our cyclic hypoxic profile (12% FiO₂) was not severe enough to prevent proper trophoblast invasion, as results in a pig model of hypoxia demonstrated that while a hypoxic exposure of 10.2% FiO₂ impaired trophoblast invasion and placental damage, while there was little or no effects when FiO₂ was 16% or 12% during pregnancy (468).

The VEGF family includes VEGF-A, B, C, D and PIGF, with three major receptors including Flt-4, KDR, and Flt-1, as well as a soluble form of Flt-1 (sFlt-1/VEGFR1) that is generated by alternative mRNA processing and which lacks both transmembrane and cytoplasmic domains (469). VEGF A, B and PIGF bind to Flt-1 as well as sFlt-1 that sequester
the ligands and inhibit their binding to their membrane receptors (470). The placenta is rich in VEGF and PIGF and their signalling is critical to the development of the placental villous angioarchitecture (471). In PE, circulating levels of VEGF and PIGF are reduced while levels of sFlt-1 and sENG are increased (472), making the sFlt-1/PIGF ratio a diagnostic predictor of PE (473). Plasma levels of sFlt-1 and sENG are elevated in pregnant patients with OSA (7) also in OSA subjects with hypertension (474) (475). We report that elevated levels of sFlt-1 and sENG in pregnant mice exposed to GIH, with simultaneous circulating levels of VEGF and PIGF-2. Overexpression of sFlt-1 can occur in response to elevated endometrial-specific VEGF production, suggesting that sFlt-1 plays an important role in maintaining vascular integrity in the placenta by sequestering excess maternal VEGF (476).

Oxidative stress describes a disturbance in the balance of the production of reactive oxygen species (ROS) and their metabolism by antioxidants (477), and is a prominent feature of OSA and its cardiovascular complications (478) in subjects (81) and animal models (122, 405). Normal gestation required ROS generation (479), but excessive ROS production occurs in PE and IUGR, where ROS likely triggers inflammation, leads to apoptosis of trophoblasts and alters vascular reactivity (480, 481). Furthermore, oxidative stress modifies post-translational modification of proteins, DNA, and lipids (482, 483). We report that GIH increases systemic levels of markers of oxidative stress (8-isoprostane) and inflammation (TNF-α). Lipid peroxidation caused by oxidative stress produces reactive aldehydes, such as 4-HNE, that can react with and deactivate a myriad of proteins and lipids and also induce apoptosis (484). We show increased immunostaining for 4-HNE adducts, with increased cell death in GIH placental sections using TUNEL staining. We also report that pimonidazole
staining was more intense in placental section from pregnant mice exposed to GIH. Of interest is the related finding that the placentas of women with OSA also have increased expression of the hypoxic marker carbonic anhydrase (CAIX) [442].

In summary, GIH can lead to fetal growth retardation and impair function of the main uterine artery. It also created an imbalance in production angiogenic and anti-angiogenic factors that may have adverse effects on pregnancy outcomes. This study provides an insight in the possible outcomes fetoplacental outcomes of pre-existing OSA in pregnant women.
Chapter 4: Gestational intermittent hypoxia induces endothelial dysfunction, reduces perivascular adiponectin and causes epigenetic changes in adult male offspring mice but not female offspring

4.1 Introduction

According to the theory of Developmental Origins of Health and Disease (DOHaD), stress during fetal development causes offspring to have subtle functional changes in specific tissues that can increase susceptibility to disease later in life (485). Epigenetic modifications are one of the main mechanisms involved in developmental programming. (486). Maternal obesity (393), gestational diabetes (487) and maternal nutrition (488) affect birth weight and metabolic profile of the offspring, and can predispose the offspring to metabolic and cardiovascular disease (489).

Obstructive sleep apnea (OSA) is characterized by recurrent collapse of the airways leading to chronic intermittent hypoxia (IH) and sleep fragmentation (2). The prevalence of OSA ranges between 13-33% in males and 6-19% in females (490). It is estimated that up to 80% of cases of moderate to severe OSA remain undiagnosed despite access to health care (491). Thus, it is likely that a great number of fetuses are subjected to gestational intermittent hypoxia (GIH) in women with pre-existing OSA, given the rise in obesity in the general population. Over one-third of women experience OSA symptoms by the third trimester with a 24% annual increase in prevalence (209). OSA is associated with preeclampsia, gestational diabetes and low birth weight (211). There is little known about the impact of OSA on the adult offspring exposed to GIH.
Endothelial dysfunction modulates the progression of atherosclerosis and cardiovascular disease (492). Affecting the maternal environment in rodents, such as by feeding with an obesogenic diet, results in offspring with endothelial dysfunction even if weaned on a standard (non-obesogenic) diet (493–495), suggesting that fetal programming causes vascular changes later in life. Perivascular adipose tissue (PVAT) modulates vascular reactivity through secretion of bioactive molecules such as adiponectin (336). PVAT has anti-contractility effects, which can be diminished by inflammation (496) and reduced adiponectin secretion (497) in healthy subjects. However, the effects of GIH on endothelial and PVAT function in the offspring from GIH are unknown.

We examined the hypothesis that GIH causes metabolic dysregulation, endothelial and PVAT dysfunction in adult offspring mice. We evaluated the effects of GIH on body weight, insulin sensitivity, endothelial function and the anti-contractile effects of PVAT. Furthermore, we assessed the role of adiponectin on the anti-contractile effects and epigenetic modifications on the adiponectin promoter in PVAT.

4.2 Materials and Methods

4.2.1 Animal experiment

Experiments were approved by the Animal Care Center at the University of British Columbia, Canada (Certificate number: A17-0140). Male and female C57BL/6J mice (8 weeks old) were purchased from Charles River (Wilmington, MA) and kept at the animal care facility in a controlled environment with 12h light/dark cycles with ad libitum access to normal chow diet and water. The breeding protocol involved by introducing two female mice
to one male mouse in each cage. After visualization of a copulation plug (day 0.5 of pregnancy), females were separated into two groups: [1] pregnant dams subjected to intermittent hypoxia (IH) and [2] pregnant dams subjected to room air (IA).

4.2.2 Intermittent hypoxia protocol

Pregnant dams were placed in specialized cages having regulated levels of gas/air inside the cages. The IH profile consists of alternating cycles of fraction of oxygen inspired ($\text{FiO}_2$) of 21-12% using nitrogen ($\text{N}_2$) and compressed air. Mice were exposed to IH for 60 cycles/h during the light cycle (12h) a day with oxyhemoglobin desaturation levels of ~75% per cycle. For the groups exposed to IA, pregnant dams were exposed to room air ($\text{FiO}_2$ 21%) only. At day 18.5 of pregnancy, IH was halted and females were separated into individual cages to deliver under normal levels of ambient air and ad libitum access to regular chow and water) (122).

4.2.3 Offspring pups

We reduced the number of pups to six after birth and these were housed with their mothers until weaned. Each litter had 2-5 males. Males and females were separated in different cages after weaning (3 weeks of age). Offspring mice had ad libitum access to water and normal chow food in a controlled environment (12h dark/light cycles and 24°C). Body weights and food consumption were measured weekly in the morning (9am). To estimate food consumption, we calculated the difference in amount of food remaining after offering a fixed amount of food for each mouse in a separate cage. Mice were sacrificed at 16 weeks
using an overdose of the inhalant anesthetic isoflurane (5% at 1-2 L\textsubscript{O}_{2}/min) followed by carbon dioxide. For all experiments, we used one offspring from each litter produced by 4-10 mothers. Plasma was collected, abdominal and thoracic aorta were excised for wire myograph studies, and PVAT and skeletal muscle tissue were stored at -80 for DNA/RNA/protein extraction.

4.2.4 Metabolic assessments

Mice were fasted for 4 hours before euthanasia. Fasting blood glucose (FBG) was measured by puncturing the medial saphenous vein and using a glucometer. Blood samples were collected to measure fasting blood insulin (FBI) using enzyme-linked immune sorbent assay (ELISA) kit (Alpco, Salem, NH). For the insulin tolerance test (ITT) (498) fasted mice were injected with insulin (0.5 U/kg of body weight of insulin) and blood glucose was measured every 15 minutes. Plasma leptin levels were measured using ELISA according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). Plasma lipid levels were measured at The Vancouver General Hospital labs.

4.2.5 Wire myography

Abdominal aortic blood vessels either cleaned of surrounding perivascular tissue or the PVAT was kept intact. Arteries were cut in equal 2mm rings and mounted on wire myograph system to measure isometric tension (DMT 620M, Danish Myotechnology, Asrhus, Denmark). Each myograph chamber contained physiologic solution (PSS) (in mM: Nacl (119), KCl (4.7), KH\textsubscript{2}PO\textsubscript{4} (1.18), MgSO\textsubscript{4} (1.17), NaHCO\textsubscript{3} (24.9), EDTA (0.023), CaCl\textsubscript{2} (1.6) and
dextrose (11.1)) kept at pH 7.4 and 37°C with administration of 95% O₂ and 5% CO₂ gases throughout the experiment. Blood vessels were stretched to their optimum tension (5.5 mN) and allowed to equilibrate for 30 minutes before constricting them with 80 mM potassium chloride solution (KCl: prepared by equimolar substitution of NaCl in PSS) and then washing with PSS to return to resting tension. For endothelium-dependent relaxation, aortic rings were constricted with a submaximal dose of phenylephrine (Ph, 1M, Sigma) followed by cumulative additions of acetylcholine (ACh, 10⁻⁹ – 10⁻⁵, Sigma) (405). For anti-contractile studies of PVAT, cumulative concentration-response curves to to the constrictor effects of U46619 (9,11-dideoxy-9a,11 a-methanoepoxy prostaglandin F₂α, Cayman Chemicals, ANN Arbor, MI) (10⁻⁹ – 10⁻⁵) was made. Some arteries were incubated for 30 minutes with mouse recombinant globular adiponectin (3 x 10⁻³ g/ml) or the adiponectin receptor 1 (AdR1) blocker (5 x 10⁻³ g/ml) (Enzo Life Sciences, Farmingdale, NY).

4.2.6 Western blotting

Skeletal muscles (lateral gastrocnemius) were snap frozen and stored at -80°C. Tissues were homogenized in RIPA buffer with protease and phosphatase inhibitors using a BeadBug homogenizer (Benchmark Scientific, Edison, NJ). Tissue homogenates were centrifuged for 10 minutes at 14,000xg and the supernatants transferred to clean tubes. The Pierce bicichoninic acid (BCA) assay was used to determine protein concentrations in skeletal muscle lysates (Thermo Fisher Scientific, Waltham, MA). For western blotting, 30μg of samples protein were loaded on polyacrylamide gels at 200 volts for 1 hour. The gels were then removed and transferred to nitrocellulose membranes overnight at 4°C using Mini-
PROTEAN Tetra cell (Bio-Rad Laboratories, Hercules, CA). Membranes were then incubated for 1 hour with 5% non-fat milk (New England Biolabs, Ipswich, MA) for blocking, and then incubated with primary antibodies in tris-buffred saline (TBS 1L: 50 mM Tris-Cl, pH 7.6; 150 mM NaCl) with 0.05% Tween-20 (Bio-Rad, Hercules, CA) (TBST) overnight at 4°C. The antibodies used were anti-Akt (also known as protein kinase B) (Cell Signaling Technology Cat# 9272, RRID: AB_329827) rabbit polyclonal IgG at a dilution of 1:1000 and anti-p-Akt at a dilution of 1:1000 (Cell Signaling Technology Cat# 9271, RRID: AB_329825). Following overnight incubation, membranes were washed for 15 minutes three times with TBS, and the secondary goat anti-rabbit horseradish peroxidase-tagged antibody was added at a dilution of 1:2000 (Cell Signaling Technology Cat# 7074, RRID: AB_2099233). After 1 hour of incubation at room temperature, detection was performed using an enhanced chemiluminescence kit Clarity Max (Bio-Rad, Hercules, CA).

4.2.7  Real time PCR for gene expression

Mature adipocytes were isolated after digesting PVAT in collagenase type I (Sigma) in Krebs-Ringer buffer to a final concentration in the solution containing PVAT plus collagenase of 1mg/ml at 37°C for 20 min. The digested tissue was filtered with a sterile 200μm filter mesh and then centrifuged at 50xg for 5 min. Mature adipocytes (white ring) from the upper part of the solution were transferred to a clean tube for RNA/DNA isolation. RNA was extracted from PVAT using using Qiazol lysis reagent (Qiagen, Hilden, Germany) and then purified using Isolated RNeasy mini kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. RNA quality and quantity were determined using Agilent 2100
Bioanalyser (Agilent Technologies, Santa Clara, CA). Total RNA with ribosomal integrity number (RIN) > 7 were used for real-time PCR quantification by custom RT² Profiler™ PCR array (Qiagen, Hilden, Germany).

Real-time PCR quantification was performed with an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) using a custom made RT² Profiler PCR array consisting of 14 SYBR® Green-optimized primer assays for 14 genes, including glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. cDNAs were synthesized from 0.5μg of total RNA using a commercial RT² First Strand Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The synthesized cDNAs were then mixed with RT² q-PCR ROX master-mix containing SYBR green (Qiagen, Hilden, Germany). The mixture was then added to the custom RT² Profiler PCR array and qPCR was performed according to the manufacturer’s instructions (Cycling conditions: 1 cycle 10 minutes 95°C to activate HotStart DNA Taq Polymerase followed by 40 cycles of 15 seconds 95°C and 1 minute 60°C). Data was analyzed using the integrated web-based automated software for RT² profiler PCR Array Data analysis (RT² profiler PCR Array Data analysis version 3.5, GeneGlobe Data Analysis, SABiosciences). Gene expression fold changes were calculated using the △△C_T method and the housekeeping gene control was used for normalization of the results. For adiponectin gene (AdipoQ) expression, the same previous procedure and reagents mentioned were used with RT² qPCR Primer Assay for Mouse AdipoQ (Qiagen, Hilden, Germany).
4.2.8 Biochemical measures

We measured plasma 4-hydroxynonenal (4-HNE, MyBioSource, San Diego, CA) as an oxidative stress marker and tumor necrosis factor-alpha (TNF-α, R&D systems, Minneapolis, MI) as an indicator of inflammation using commercially available ELISA kits. We also used ELISA kits for measuring adiponectin levels in both plasma and PVAT tissue homogenate. All assays were performed according to the manufacturer’s instructions.

4.2.9 DNA methylation using pyrosequencing method

We used PyroMark Assay Design 2.0 (Qiagen, Hilden, Germany) software to design bisulfite pyrosequencing assays covering the adiponectin promoter. Genomic DNA samples from PVAT mature adipocytes were bisulfite converted using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA) following the manufacturer’s protocol. Two separate amplicons (R1: 2 CpG loci 500-600bps upstream of the transcription start site; R2: 4 CpG loci ~1kb upstream of the transcription start site) were generated by PCR using biotinylated primers (IDT, San Jose, CA; see Table for sequences) and each bisulfite converted samples. The regions of interest were amplified by PCR using the HotstaTaq DNA polymerase kit (Qiagen, Inc.) as follows: 15 minutes at 95°C (to activate the Taq polymerase), 45 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, and a 5-minute 72°C extension step. Streptavidin-coated sepharose beads were bound to the biotinylated strand of the PCR product and were then washed and denatured to yield single-stranded DNA using Pyromark Vacuum Prep Workstation (Qiagen, Inc.). Sequencing primers were then added for pyrosequencing as per manufacturer’s instructions (Pyromark™ Q96 MD Pyrosequencer,
Qiagen, Inc.) (499). The quantitative levels of methylation for each CpG dinucleotide were calculated with Pyro Q-CpG software (Qiagen, Inc.)

Table 4-1: Oligos sequences used for pyrosequencing Adiponectin promoter CpGs

<table>
<thead>
<tr>
<th>Oligo Name</th>
<th>Sequence (5' &gt; 3')</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1 - forward</td>
<td>GTTGGTTGTTAGTTTAGTTAATAATAGA</td>
<td>Amplicon generation</td>
</tr>
<tr>
<td>R1 - reverse (5' Biotinylated)</td>
<td>ATCCTAAATTCAATTCACCACAC</td>
<td>Amplicon generation</td>
</tr>
<tr>
<td>R1 - Sequencing primer 1</td>
<td>GTTTTTTTTTTAGTGATGGGA</td>
<td></td>
</tr>
<tr>
<td>R1 - Sequencing primer 2</td>
<td>ATGTATATTTGTATATTTAAAAGAAGG</td>
<td></td>
</tr>
<tr>
<td>R2 - forward</td>
<td>GGTAGTGGAGGAAGTAGATGTTTG</td>
<td>Amplicon generation</td>
</tr>
<tr>
<td>R2 - reverse (5' Biotinylated)</td>
<td>CCTACATAATTCAACCCCAATATCACAA</td>
<td>Amplicon generation</td>
</tr>
<tr>
<td>R2 - Sequencing primer 1</td>
<td>GGTATTTAGAGTTTTTTTTTGGAT</td>
<td></td>
</tr>
<tr>
<td>R2 - Sequencing primer 2</td>
<td>AGTTTTGGTTTTTTAAAAATA</td>
<td></td>
</tr>
</tbody>
</table>

4.2.10 Statistical analysis

All data are reported as the mean ± SD, where n represents one offspring from each litter. Comparisons of numerical data were performed using Student's unpaired t test and two-way ANOVA with repeated measures, where a P-value <0.05 was considered as statistically significant.
4.3 Results

4.3.1 Low birth weights followed by increased weight gains in male pups exposed to GIH

There was a significant interaction between GIH exposure and body weights \((P<0.0001)\), and food consumption \((P<0.0001)\) in both male and female mice. Male pups from the IH group had lower weights at week 1 compared to pups from the IA group \((2.7 \pm 0.2g \text{ vs. } 3.6 \pm 0.3g, P<0.05)\) (Figure 4-1A). IH pup weights remained significantly lower until week 6 in male pups, but gained weight compared to IA pups from weeks 9 to 16 \((39.9 \pm 1.2g \text{ vs. } 32.3 \pm 0.7g, P<0.05)\). Food consumption increased significantly in the IH group after week 7 \((P<0.05)\) (Figure 4-1B). Female pups’ weights were lower for the first 3 weeks of age when compared to control group then there was no significant difference until week 16 (Figure 4-1C). Food consumption was not significantly different in female pups from both groups (Figure 4-1B).
Figure 4-1: Low birth weights followed by later increased body weights and food consumption of male pups exposed to GIH.

Body weights from week 1 to 16 of age (A, C) and food consumption from week 3 (weaning) to 16 (B, D) in pups exposed to GIH or IA. Values are displayed as mean ± SD and represent n = 10 mice. Groups were compared using two way ANOVA with repeated measures. *P<0.05 vs. IA.
4.3.2 GIH induces dyslipidemia, hyperleptinemia and insulin resistance in male pups

Visceral and subcutaneous fat pads weights were ~2 fold higher in IH male pups as were plasma levels of triglycerides, cholesterol and free fatty acids (FFA) \( P < 0.05 \) (Table 4-2). Fasting blood glucose, insulin and leptin levels were also higher in IH male pups \( P < 0.05 \). Furthermore, insulin tolerance to exogenous insulin was impaired in IH male pups (% of basal glucose: \( 33.9 \pm 6.7\% \) vs. \( 73.5 \pm 4.1\% \), \( P < 0.05 \)) (Figure 4-2A) and the protein expression ratio of p-Akt: total Akt in skeletal muscle (an indicator of tissue specific insulin resistance) was also higher in IH male pups \( (0.37 \pm 0.16 \) vs. \( 0.65 \pm 0.16 \), \( P < 0.05 \)) (Figure 4-2B). There were no differences in lipid profiles or fasting levels of blood glucose or insulin when compared to IA pups (Table 4-2).
### Table 4-2: Metabolic parameters of IA and IH pups at weeks 16 of age

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Male</th>
<th>Female</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IA</td>
<td>IH</td>
<td>IA</td>
<td>IH</td>
</tr>
<tr>
<td>Visceral fat (% body weight)</td>
<td>1.7 ± 0.1</td>
<td>2.5 ± 0.1*</td>
<td>0.79 ± 0.07</td>
<td>0.82 ± 0.06</td>
</tr>
<tr>
<td>Subcutaneous fat (% body weight)</td>
<td>0.87 ± 0.03</td>
<td>1.54 ± 0.05*</td>
<td>0.45 ± 0.05</td>
<td>0.46 ± 0.05</td>
</tr>
<tr>
<td>Adiposity index (%)</td>
<td>2.6 ± 0.1</td>
<td>4.1 ± 0.1*</td>
<td>1.2 ± 0.1</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>1.11 ± 0.07</td>
<td>1.69 ± 0.14*</td>
<td>0.95 ± 0.15</td>
<td>0.94 ± 0.12</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>2.19 ± 0.26</td>
<td>3.52 ± 0.25*</td>
<td>2.04 ± 0.16</td>
<td>2.14 ± 0.21</td>
</tr>
<tr>
<td>Free Fatty Acids (mmol/L)</td>
<td>0.52 ± 0.04</td>
<td>0.87 ± 0.03*</td>
<td>0.43 ± 0.07</td>
<td>0.51 ± 0.08</td>
</tr>
<tr>
<td>Fasting blood glucose (mmol/L)</td>
<td>3.8 ± 0.3</td>
<td>7.6 ± 0.9*</td>
<td>3.9 ± 0.5</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>Fasting blood insulin (pg/ml)</td>
<td>0.35 ± 0.02</td>
<td>0.65 ± 0.06*</td>
<td>0.34 ± 0.04</td>
<td>0.37 ± 0.04</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>5.3 ± 1.4</td>
<td>59.2 ± 16.4*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are reported as mean ± SD and represent n = 10 mice. Groups were compared using Student’s unpaired t-test. *P<0.05 vs. IA.
Figure 4-2: Increased insulin resistance in male pups exposed to GIH.

Insulin tolerance test (A) and ratio of protein expression of p-Akt/Akt in skeletal muscle tissue (B) from IH and IA male pups. Values are displayed as mean ± SD and represent n = 5-10 mice. Groups were compared using Student’s unpaired t-test. *P<0.05 vs. IA. Akt: protein kinase B, p-: phosphorylated, GAPDH: glyceraldehyde 3-phosphate dehydrogenase
4.3.3 Improved endothelium-dependent relaxation in male pups exposed to GIH

Aortic blood vessels response to ACh was impaired in IH male pups (Figure 4-3A), with a lower maximal response to ACh ($E_{\text{max}}$: 69.2 ± 5.4% vs. 90.1 ± 5.6%, $P < 0.05$) (Figure 4-3B). There were no differences in response to SNP ($P = \text{NS}$) (Figure 4-3C). In IH female pups, relaxation responses and maximal responses to ACh were similar to IA female pups (Figure 4-3C, D).
Figure 4-3: Endothelial dysfunction in male pups exposed to GIH.

Concentration-response curves (A, C) and maximal responses (B, D) to ACh, and concentration-response curves to SNP (C) from IH and IA pups. Values are displayed as mean ± SD and represent n = 5-6 mice. Groups were compared using Student’s unpaired t-test. *P<0.05 vs. IA. ACh: acetylcholine, SNP: sodium nitroprusside
4.3.4 Loss of anti-contractile response in male pups exposed to GIH

The contractile responses to U46619 in the intact aorta (i.e. with PVAT present) from IA male pups was reduced when compared to PVAT-denuded arteries ($E_{max}$ %KPSS: $83.1 \pm 12.3\%$ vs. $126.4 \pm 11.8\%, P < 0.05$) (Figure 4-4A). There were no differences in response to U46619 between PVAT intact and denuded arteries in IH male pups ($P = NS$) (Figure 4-4B), indicating PVAT modulates vascular contractility in IH pups.
Figure 4-4: Reduced PVAT anti-contractile activity in male pups exposed to GIH

Concentration-response curves to U46619 in PVAT-denuded and PVAT-intact abdominal aortic arteries in IA (A) and IH (B) male pups. Values are displayed as mean ± SD and represent n = 5-6 mice. Groups were compared using Student’s unpaired t-test. *P<0.05 vs. IA. PVAT: perivascular adipose tissue, U46619: 9,11-dideoxy-9a,11a-methanoepoxy prostaglandin F₂α
4.3.5 Exogenous adiponectin administration restores anticontractile activity in male pups exposed to GIH

Incubating intact aortic blood vessels from IA male pups with globular adiponectin (gAd, $3 \times 10^{-3}$ g/ml) produced no additional anti- contractile effects ($P = NS$) (Figure 4-5A). However, adding gAd to PVAT-denuded arteries produced anti- contractile effects in tissues from IA male pups ($E_{max} \%$KPSS: $98.1 \pm 5.5\%$ vs. $126.4 \pm 9.6\%, P < 0.05$) (Figure 4-5B). Addition of gAd to the intact aorta from IH male pups reduced the contractile response to U46619 ($E_{max} \%$KPSS: $94.2 \pm 13.2\%$ vs. $140.6 \pm 19.4\%, P < 0.05$) (Figure 4-5C). Furthermore, incubation with gAd reduced the contractile response to U46619 in PVAT-denuded arteries ($E_{max} \%$KPSS: $104.4 \pm 6.1\%$ vs. $147.4 \pm 19.4\%, P < 0.05$) (Figure 4-5D).
Figure 4-5: Adiponectin restored anti-contractile activity in male pups exposed to GIH.

Concentration response curves to U46619 after incubation with gAd in PVAT-intact (A) and PVAT-denuded (B) abdominal aortic arteries from IA male pups, and PVAT-intact (C) and PVAT-denuded (D) abdominal aortic arteries from IH male pups. Values are displayed as mean ± SD and represent n = 5-6 mice. Groups were compared using Student’s unpaired t-test. *P<0.05 vs. IA. gAd: globular adiponectin, PVAT: perivascular adipose tissue, U46619: 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin F2α
4.3.6 Adiponectin is essential for the anti-contractile activity in PVAT

Incubation of PVAT-intact aortic blood vessels from IA male pups with an AdR1 blocker (Blocking peptide for AdR1, $5 \times 10^{-3}$ g/ml) reduced the anti-contractile response to U46619 ($E_{max} \%KPSS: 83.1 \pm 12.3\%$ vs. $124.5 \pm 10.6\%$, %, $P < 0.05$) (Figure 4-6A). However, the AdR1 blocker did not alter the anti-contractile effects of PVAT in IH pups ($P = NS$) (Figure 4-6B).
Figure 4-6: Blocking AdR1 in PVAT abolished anti-contractile activity.

Concentration-response curves to U46619 after incubation with AdR1 blocker in abdominal aortic arteries from IA (A) and IH (B) male pups. Values are displayed as mean ± SD and represent n = 5-6 mice. Groups were compared using Student’s unpaired t-test. *P<0.05 vs. IA. AdR1: adiponectin receptor 1 blocker, PVAT: perivascular adipose tissue, U46619: 9,11-dideoxy-9a,11a-methanoepoxy prostaglandin F2α
4.3.7 Lower adiponectin levels in plasma and PVAT in male pups exposed to GIH

Adiponectin plasma levels were lower in IH male pups (Table 4-3). Tissue levels of adiponectin levels in PVAT were also lower in IH male pups accompanied by lower adiponectin gene expression from extracted RNA (Table 4-3).
Table 4-3: Circulating and PVAT adiponectin levels in male pups

<table>
<thead>
<tr>
<th>Adiponectin Levels</th>
<th>IA</th>
<th>IH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma (μg ml⁻¹)</td>
<td>25 ± 2.7</td>
<td>15.4 ± 1.5*</td>
</tr>
<tr>
<td>PVAT (ng ml⁻¹ in 20mg)</td>
<td>18 ± 0.1</td>
<td>11 ± 0.1*</td>
</tr>
<tr>
<td>PVAT RNA (fold of IA in PVAT)</td>
<td>1.00 ± 0.12</td>
<td>-2.86 ± 0.34*</td>
</tr>
</tbody>
</table>

Values are displayed as mean ± SD and represent n = 8 mice. Groups were compared using Student's unpaired t-test. *P<0.05 vs. IA. PVAT: perivascular adipose tissue
4.3.8 **Increased systemic oxidative stress, inflammation, and inflammatory gene expression in PVAT of male pups exposed to GIH**

Plasma levels of the oxidative stress marker 4-HNE were higher in IH male pups (26.3 ± 1.6ng/ml vs. 10.5 ± 1.8ng/ml, \( P < 0.05 \)) (Figure 4-7A). Moreover, plasma levels of the inflammatory cytokine TNF-\( \alpha \) plasma were higher in male pups from mothers with GIH (25.5 ± 6.9pg/ml vs. 9.9 ± 2.9pg/ml, \( P < 0.05 \)) (Figure 4-7B). Gene expression of inflammatory markers such as interleukin 6 (IL6), monocyte chemoattractant protein 1 (Ccl2), interferon gamma (Ifng) and Tnf were ≈3 to 8 folds higher in PVAT of male pups from mothers with GIH (\( P < 0.05 \)) (Figure 4-7C). Gene expression of vascular cell adhesion molecule 1 (Vcam1) but not of intracellular adhesion molecule 1 (Icam1) was significantly higher in PVAT of male pups from mothers with GIH (\( P < 0.05 \)). The gene expression of antioxidant enzymes such as superoxide dismutase 1 (Sod1), glutathione peroxidase 3 (Gpx3), heme oxygenase 1 (Hmox1) and reactive oxygen species (ROS) producing enzymes such as NADPH oxidase 1 & 4 (Nox1, Nox4) in PVAT were not significantly different between male pups from mothers with either GIH or IA (\( P = \text{NS} \)). However, the gene expression of the antioxidant enzyme NAD (P) H dehydrogenase was ≈3.5 fold higher in male pups from mothers with GIH (\( P < 0.05 \)).
Figure 4-7: Systemic oxidative stress, inflammation and pro-inflammatory gene expression is increased in male pups exposed to GIH

Plasma 4-HNE (A) and TNF-α (B) in male pups. Fold regulation (C) of gene expression of inflammatory cytokines, antioxidant enzymes, adhesion molecules and ROS-producing enzymes in PVAT of male pups. Values are displayed as mean ± SD and represent n = 8 mice. ΔΔC_T values between groups were compared using Student’s unpaired t-test. *P<0.05 vs. IA. Fold regulation represents fold-change in a biologically meaningful way. 4-HNE: 4-hydroxynonenal, IL-6: Interleukin 6, TNF-α: Tumor necrosis factor-α, Mcp1: Monocyte chemoattractant protein 1, Ifng: Interferon gamma, Icam1: Intracellular adhesion molecule 1, Vcam1: Vascular cell adhesion molecule 1, Sod1: Superoxide dismutase 1, Gpx3: Glutathione peroxidase 3, Hmox1: Heme oxygenase 1, Nqo1: NAD(P)H dehydrogenase, Nox1: NADPH oxidase 1, Nox4: NADPH oxidase 4
4.3.9  **Hypermethylation of adiponectin gene promoter in male pups exposed to GIH**

The percentage of DNA methylation of CpG islands of adiponectin gene promoter in PVAT of male pups from mothers with GIH was higher at positions 1 (74.1 ± 2.7% vs. 61.5 ± 1.9%, \( P < 0.05 \)), 17 (72.6 ± 6.8% vs. 62.7 ± 4%, \( P < 0.05 \)), 12 (48.4 ± 6.5% vs. 38.7 ± 2.1%, \( P < 0.05 \)) and 14 (60.9 ± 9.1% vs. 43.4 ± 1.1%, \( P < 0.05 \)) (Figure 4-8C). The DNA methylation at positions 14 and 15 were similar in PVAT from male pups from mothers with either GIH or IA (\( P = \text{NS} \)).
Figure 8: Increased DNA methylation of adiponectin gene promoter in PVAT of male pups exposed to GIH

Schematic overview of the CpG dinucleotide regions on the adiponectin promoter (A). Selected CpG dinucleotide regions on adiponectin promoter sequence (B) and percentage of methylation of different CpGs on adiponectin gene promoter (C). Values are displayed as mean ± SD and represent n = 4-5 mice. Groups were compared using Student’s unpaired t-test. *P<0.05 vs. IA.
4.4 Discussion

We examined the effects of GIH on metabolic and vascular function in adult male mice offspring and show that GIH can lead to: 1) low birth weights and later increases in body weights and food consumption, 2) dyslipidemia, hyperleptinemia and insulin resistance, 3) increased systemic oxidative stress, inflammation, 4) impaired endothelial function in the abdominal aorta, 5) loss of anti-contractile activity of PVAT, 6) low circulating and PVAT adiponectin levels, and increased inflammatory gene expression in PVAT, and 7) hypermethylation of adiponectin gene promoter. A summary of these findings is depicted in Figure 9. Female pups did not exhibit any difference in weights after 3 weeks, food consumption, lipid profile, fasting insulin and glucose levels or endothelial function at week 16. Our data is supported by other studies on sexual dimorphism in terms of responses to perturbations in utero (500–502).
Figure 4-8: Cardiometabolic consequences of GIH in adult male mouse offspring

The prevalence of OSA continues to escalate, and is in part fueled by the growing global obesity epidemic (503). Over one-third of women experience OSA symptoms by the third trimester with an annual increase in prevalence of 24% (209). OSA during pregnancy is associated with preeclampsia (504), gestational diabetes (223) and reduced fetal growth (222). However, the effect of pre-existing OSA during pregnancy on fetal/newborn health is unknown.

Our studies agree with the findings of a recent study by Khalyfa et al reporting that late GIH in mice (days 13-18 of gestation) experiencing severe IH (FiO₂ 21 to 6.1%) increased body weights at 24 weeks of age, and metabolic dysregulation (increased weight gains, insulin resistance, dyslipidemia and hyperleptinemia) through epigenetic modifications in visceral white adipose tissue in male but not female offspring mice (505). The study by Khalyfa et al did not examine cardiovascular function in offspring mice; in their study, late GIH did not result in low birth weights, and another important difference is that we induced IH at day 1 of pregnancy using a less severe IH profile (FiO₂ 21 to 12%). The timing of maternal insults is important in determining long-term outcomes in the offspring (506). For instance, exposure to famine early in the gestational period in humans (1-10 weeks), but not during the mid to late gestational period, results in adult DNA methylation changes in whole blood (507).

Growth restriction in humans early in gestation increases the probability of developing obesity later in life, especially if combined with rapid compensatory growth after birth (508). For instance, low birth weight increases food intake in school-aged boys (509). Maternal nutrient deficiency and maternal obesity in animals increases obesity rates in the
offspring during adulthood (510, 511). Nutrient deprivation and overnutrition of mice increases hyperphagia as a result of leptin resistance in the offspring (512, 513), with proposed mechanisms including impaired transport of leptin across blood-brain barrier (514) and increased production of orexigenic neuropeptides such as galanin and enkephalin in developing pups (515). We show that pups from mothers experiencing GIH had low birth weights that were later followed by increased body weights, food consumption and hyperleptinemia.

Impaired endothelium-dependent relaxation occurs in children (9-11 years) (516) and young adults (20-28 years) born after fetal growth restriction (517). Low-birth weights of offspring in many animal models impair NO-dependent vasodilation in aorta (518) and small arteries (519) including coronary arteries (520). Four-month-old offspring of pregnant rats exposed to chronic hypoxia exhibit impairs NO-dependent relaxation in femoral resistance arteries, which was prevented by maternal vitamin C treatment, suggesting that oxidative stress plays an important role in developmental programming of cardiovascular disease (306). Late gestational hypoxia in dams combined with a postnatal high salt diet causes endothelial dysfunction in mesenteric arteries and increased vascular stiffness in both males and female offspring at 12 months of age (521). Our study shows that male, but not female, pups exposed to GIH had impaired aortic NO-dependent vasodilation at week 16 of age.

PVAT has important roles in mediating vascular function, intravascular thermoregulation, vascular inflammation, vascular smooth muscle cells proliferation and the development of atherosclerosis (336). PVAT influences vascular reactivity through
endocrine and paracrine effects by releasing biologically active molecules including perivascular-derived relaxing factors (PVRF) (522). These mediators that are responsible for the anti-contractile function in PVAT. The identity of PVAT is unknown but suggested molecules include: hydrogen peroxide (H2O2), hydrogen sulphate (H2S), angiotensin 1-7 and angiotensin II, nitric oxide (NO), methyl palmitate, leptin and adiponectin (336, 523). Adiponectin has significant anti-contractile activity in PVAT, as suggested by the reduced anti-contractile activity of PVAT in adiponectin-deficient mice (330). Blocking adiponectin receptors inhibits the anticontractile activity of PVAT (331). It has been proposed that adiponectin induces anti-contractile effects through many mechanisms including hyperpolarization of VSMC, stimulating NO release and enhancing endothelial NO production (336). Moreover, adiponectin reduces vascular oxidative stress by inhibiting NADPH oxidase (335). We show that the anti-contractile activity of PVAT was absent in pups from mothers exposed to GIH, in keeping with other studies of a loss of anti-contractile activity in PVAT-intact mesenteric arteries from male rat pups of dams a fed high fat diet at 12 and 24 weeks of age (341). Incubation with an AdR1 blocker abolished the anti-contractile activity in IA male pups. We show that circulating and PVAT homogenate levels of adiponectin as well as adiponectin gene expression in PVAT were significantly lower in IH male pups.

Local inflammation has an important role in PVAT dysfunction, as shown in apolipoprotein E -/- (Apoe-/-) mice where PVAT inflammation preceded atherosclerotic plaque formation and endothelial dysfunction (524). Dysfunctional PVAT obtained from animal models with obesity increase the expression of inflammatory cytokines and
chemokines such as Tnf, Ccl2, Il6 and Ifng (337, 525). Chronic adiponectin administration to rats fed a high-fat diet improves NO-dependent vasorelaxation and reduces the expression of chemokines and pro-inflammatory adipokines in PVAT (325). We show that GIH increased PVAT gene expression of inflammatory cytokines accompanied by low adiponectin gene expression, suggesting that low adiponectin levels are associated with PVAT inflammation.

Epigenetic modifications are proposed as key mechanisms in developmental programing of the offspring (526). DNA methylation, a prominent epigenetic process, is often linked to decreased gene expression in living systems (527). A study by Khalyfa et al reported that late GIH caused epigenetic alterations and increased DNA methylation in visceral white adipose tissue, which was associated with metabolic dysregulation and obesity in male pups (505). We hypothesized that the low adiponectin levels in PVAT is associated with epigenetic modifications of the adiponectin gene promoter, and demonstrate that the adiponectin promoter was hypermethylated in IH male pups PVAT—suggesting that this may account, at least in part, for the decreased adiponectin gene expression we observed.

In summary, GIH can lead to low birth weight followed by catch-up growth in male offspring, which has been suggested to correlate with the risk of future cardiometabolic disease. Male, but not female, offspring from mothers with GIH exhibited metabolic syndrome with vascular and perivascular dysfunction. One can speculate that treating women with OSA before and during pregnancy with CPAP and following up with their offspring may prevent future cardiometabolic disease in the offspring.
Chapter 5: Conclusions

5.1 Overall

The data presented in this thesis addresses the aims set out for the study in mice exposed to CIH (a mouse model of OSA):

- To evaluate vascular outcomes after treatment with ALA
- To assess uterine artery function and feto-placental consequences during pregnancy
- To determine the cardiometabolic outcomes in the offspring

In fulfilling our aims, these studies allowed us to address the hypotheses that:

- ALA treatment mitigates endothelial dysfunction, oxidative stress and inflammation in mice exposed to CIH.
- GIH impairs uterine artery function and leads to fetal growth restriction in pregnant mice
- Offspring of dams exposed to GIH have adverse cardiometabolic disease later in adulthood through epigenetic modifications.

Our findings propose a potential therapeutic agent for treating the underlying mechanisms of CVD in OSA patients. They also expand our knowledge on the potential adverse effects of pre-existing OSA on maternal/fetal outcomes, and address aspects of developmental programming of cardiometabolic disease in OSA. This work adds to our understanding of the complex pathophysiology of OSA and its potential treatments. The implications, strengths, and limitations of our studies are described below.
5.2 Therapeutic potential of ALA in OSA

In Chapter 1, we demonstrated that CIH increases systemic and vascular oxidative stress and inflammation, leading to reduced nitric oxide bioavailability and endothelial dysfunction. Treating mice with the antioxidant ALA preserved NO-dependent dilation and reduced inflammation and oxidative stress, suggesting that ALA may be a potential therapeutic agent for improving cardiovascular outcomes in OSA patients, especially after the emerging evidence on the ineffectiveness of traditional therapy with CPAP, likely due to poor patient compliance (62, 63). In addition to ALA’s large therapeutic window and minimal side effects, the variety of formulations, its low pricing, and over-the-counter availability makes it a desirable treatment option for OSA patients (166). However, clinical trials of humans with OSA are needed to confirm our results. In Table 5-1, the strengths and limitations of our model are described. While the findings in this study provide rich insight into effectiveness of ALA, our study has limitations. First, we did not measure plasma concentrations of ALA although previous studies show that subjects taking 1200mg of LA achieve comparable serum $C_{max}$ and AUC measured in mice receiving a 50mg/kg subcutaneous dose of ALA (528). Second, treatment with ALA was initiated at the time of the CIH insult and so studies prevention (and not treatment) of endothelium-dysfunction, oxidative stress and inflammation. Not all OSA patients are diagnosed at the onset of OSA and it is possible that treatment may not the reverse the cardiovascular outcomes seen in our study. For instance, a recent study reported that short term (10 days) and long term (30 days) IH caused increased ROS levels in rat carotid body and adrenal medulla. Interestingly, ROS levels normalized after cessation of IH in the short (10 days) but not long term (30 days)
(529). Finally, ALA had prooxidant effects in the control group (IALA) as manifested by reduced NO-dependent dilation. Reports in the literature suggests that some ALAs can act as prooxidants depending on the type of oxidant stress and the physiological conditions (408) while other studies suggest an interference with the physiological role of ROS signal transduction, and favoring the production of peroxynitrite (a strong oxidant causing endothelial dysfunction) (530). In such cases, ALA might be harmful in healthy subjects.

Table 5-1: Strengths and limitations of the mouse model of OSA

<table>
<thead>
<tr>
<th>Strengths</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Mimics CIH and sleep fragmentation in OSA patients</td>
<td>• Does not incorporate airway obstruction as the main factor in altering arterial blood-gases levels during sleep</td>
</tr>
<tr>
<td>• Can be modified to resemble a wide range of IH profiles seen in OSA patients (Mild – Severe)</td>
<td>• Absence of hypercapnia and large negative intrathoracic pressure swings that could alter systemic hemodynamics</td>
</tr>
<tr>
<td>• Allows the application of genetic approaches in elucidating pathophysiology of CIH</td>
<td>• Can only focus on mechanisms of pathophysiology sequelaes rather than pathogenesis of upper airway obstruction in OSA</td>
</tr>
<tr>
<td>• Eliminates the effect of confounding factors found in most OSA patients (e.g obesity, diabetes)</td>
<td></td>
</tr>
<tr>
<td>• Very well-characterized model and extensively used</td>
<td></td>
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</tbody>
</table>
5.3 Insight into pathophysiology of OSA in pregnancy

After providing evidence of adverse vascular outcomes and exacerbated oxidative stress and inflammation caused by CIH in Chapter 1, we hypothesized that gestational IH could produce similar outcomes in pregnant mice. In Chapter 2, our study demonstrated that GIH impaired uterine artery endothelium-dependent dilation and increased contractility responses, which could reduce blood flow to the feto-placental unit in vivo (531). GIH also caused placental oxidative stress and hypoxia; two insults known to cause placental dysfunction, which can lead to fetal growth retardation and developmental programming of disease later in life (233). We also report an imbalance in the production of angiogenic and anti-angiogenic factors in GIH mice, which can lead to placental dysfunction and complications (532).

Although OSA is associated with adverse maternal and fetal outcomes, there is no data on pregnancy outcomes in women previously diagnosed with OSA. The outcomes of our study may alert medical practitioners and researchers to the potential threat of pre-existing OSA to pregnant women health and their fetuses. Women at risk of developing OSA (eg. related to obesity) should be assessed for OSA before pregnancy and monitored during gestation with tailored CPAP treatment, since pregnancy can worsen OSA outcomes towards the third trimester (229).

A limitation of our study, as mentioned in Table 1-6, is that it is technically challenging to image mouse placenta and fetus. It is also important to consider fundamental differences in mouse and human pregnancies in terms of anatomical and physiological characteristics such as gestation period, uterine shape, number of embryos, number and role of spiral
arteries, and the site of generation of progesterone in late pregnancy (533). Another limitation of our methodology is that we did not measure blood pressure in our experiments, as some of the pathological outcomes described in this study may be secondary to hypertension.

5.4 Detrimental effects of OSA on the next generation

The data in Chapter 4 suggests that an atypical maternal environment, such as GIH, can result in a phenotypic plasticity in offspring mice, which result in detrimental cardiometabolic outcomes later in life in a sex-specific manner. We show that pups exposed to GIH have low birth weights with later catch-up growth and increased body weights only in male offspring. Male offspring from mothers with GIH developed metabolic syndrome and exhibited impaired endothelium-dependent relaxation later in life, even though they were exposed to normoxia and a normal diet after weaning. Our data supports the notion that metabolic and cardiovascular dysfunction associated with GIH has developmental origins. Based on this, one might predict that offspring of mothers with pre-existing OSA could suffer from cardiometabolic disturbances early in life. This necessitates the importance of proper early interventions in women of reproductive age at risk of OSA, monitoring their offspring especially if they were born small for gestational age.

Our study also highlights the importance of the role of adiponectin in maintaining vascular homeostasis by PVAT. We show that offspring from mothers with GIH have epigenetic alterations in the adiponectin gene promoter region are associated with reduced expression in PVAT, so identifying adiponectin as a potential therapeutic target in future
vascular studies in offspring of mothers with OSA. In support of this suggestion is a study reporting that adiponectin supplementation of obese pregnant mice restored maternal insulin sensitivity, protected placental function, and normalized fetal growth (534).

Our study has some limitations; first, we only examined offspring at 16 weeks old, suggesting that metabolic syndrome as may be a pathological variable contributing to the vascular disease in our study, and second, we did not assess PVAT dysfunction adiponectin promoter methylation in PVAT in female mice. Nevertheless, our study is the first to implicate developmental cardiovascular programming of GIH in a mouse model of OSA.

5.5 Future directions

Additional studies are required to further understand a number of factors that could provide better insights of CIH and understanding its detrimental effects of GIH. The following are suggested as potential future studies:

1. Designing an experiment where mice are exposed to CIH for a long period of time (e.g. two months) before initiating treatment with dietary ALA or cessation of CIH for two months. The results will reflect whether ALA and/or cessation of CIH can reverse vascular dysfunction oxidative stress and inflammation in mice. Assessing other oxidative stress and inflammatory markers that contribute significantly to atherosclerosis such as 3-nitrotyrosine (product of peroxynitrite) and oxidized LDL and it vascular receptor (LOX) receptor expression. Moreover, assessing vascular morphometry and endothelial function in resistance vessels can be more relevant in evaluating vascular injury in response to CIH.
2. Additional experiments to elucidate the cellular mechanisms by which CIH induces vascular dysfunction. Protein, RNA and DNA microarrays are promising tools in this context. Results from such experiments may reveal new mechanisms and potential therapeutic targets.

3. Assessing uterine and umbilical artery blood flow using ultrasound to better evaluate feto-placental circulation (447) in pregnant mice exposed to GIH. Blood pressure measurements using telemetry in mice exposed to CIH (and also in mothers with GIH) are essential to determine a primary or secondary role of hypertension (535). Assessing pimonidazole immunostaining in fetal tissues may provide evidence that hypoxia also occurs in the fetuses.

4. Evaluating the effects of GIH on male vs. female placentas in Chapter 3 may provide more insight in the sexual dimorphism seen in adult male and female pups in Chapter 4.

5. Since treatment of mice exposed to CIH with ALA improved vascular outcomes and ameliorated oxidative stress and inflammation, the use of ALA may prove beneficial in improving uterine artery function and mitigating placental oxidative stress (oral ALA is safe for use in human pregnancy with no adverse outcomes in mothers or newborn (536)). As discussed in Chapter 1, hypoxia-mediated oxidative stress could be responsible for developmental programming of CVD in the offspring. Maternal antioxidant treatments (such as resveratrol) has been shown to prevent the detrimental effects of oxidative stress (361). Studying the
effects of ALA on vascular and metabolic outcomes in offspring of dams exposed to GIH could provide useful additional insights.

6. It is important to assess cardiovascular function and metabolic parameters in offspring of dams exposed to GIH at varying points in time during development. As reported in Chapter 4, male offspring had catch up growth at 8 weeks of age, at which point they gained weight. Early non-pharmacological interventions such as calorie restriction and/or exercise could prevent cardiometabolic disease in the offspring of mothers with GIH (537).

7. Evaluating other epigenetic processes such as histone modifications and miRNAs may be helpful in understanding the epigenetic processes involved in PVAT dysfunction in Chapter 4. Performing whole genome methylation sequencing can lead to identifying new therapeutic targets.

8. Studying the effect of maternal GIH on life span and cognitive functions of offspring mice may provide additional insight into the pathophysiology of gestational OSA.
5.6 Final thoughts

The prevalence of OSA is on the rise and the majority of patients who suffer from OSA are still undiagnosed. Conventional therapies, such as CPAP, are expensive, undesirable, and of limited effectiveness in preventing cardiovascular disease. There is clearly a need to pursue alternative treatments that are more convenient and effective. The pathophysiology of OSA in the pregnant population and their progeny is still unexplored despite the substantial progression in the OSA field and considering the advancement of current research tools. The outcomes of this thesis open a new frontier in identifying therapeutic candidates for OSA management and providing insight in the pathophysiology of OSA in pregnancy that may translate into cardiometabolic disease in the adult offspring.
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